UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Zoología y Antropología Física

TESIS DOCTORAL

Diversidad y especificidad de simbiontes en aves neotropicales

Diversity and host specificity of symbionts in neotropical birds

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Michaël André Jean Moens

Directores

Javier Pérez Tris
Laura Benítez Rico

Madrid, 2017

Diversidad y Especificidad de Simbiontes en Aves Neotropicales

(Diversity and Host Specificity of Symbionts in Neotropical birds.)

Tesis doctoral de:

Michaël André Jean Moens

Directores :

Javier Pérez Tris

Laura Benítez Rico

Madrid, 2016

UNIVERSIDAD COMPLUTENSE MADRID

Cover Front

Chestnut-breasted Coronet (*Boissoneaua matthewsii*)

Taken at the San Isidro Reserve, Ecuador.

Cover Back

Royal Flycatcher (*Onychorhynchus coronatus*)

Taken at the Nouragues Reserve, French Guiana.

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS
DEPARTAMENTO DE ZOOLOGÍA Y ANTROPOLOGÍA FÍSICA

Diversidad y Especificidad de Simbiontes en Aves Neotropicales

(Diversity and Host Specificity of Symbionts in Neotropical birds.)

Tesis doctoral de:

Michaël André Jean Moens

Directores:

Javier Pérez Tris

Laura Benítez Rico

Madrid, 2016

UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS
DEPARTAMENTO DE ZOOLOGÍA Y ANTROPOLOGÍA FÍSICA

Diversidad y Especificidad de Simbiontes en Aves Neotropicales

(Diversity and Host Specificity of Symbionts in Neotropical birds.)

Memoria presentada por Michaël André Jean Moens para optar al grado de Doctor en Ciencias Biológicas, bajo la dirección del Doctor Javier Pérez Tris y la Doctora Laura Benítez Rico, de la Universidad Complutense de Madrid.

Madrid, 2016

El doctorando	*Vº Bº del director*	*Vº Bº del director*
Michaël André Jean Moens	Javier Pérez Tris	Laura Benítez Rico

La presente Tesis Doctoral ha sido financiada por una beca predoctoral de Formación de Personal Investigador (FPI) concedida por el Ministerio de Ciencia e Innovación (BES-2011-047609) y era asociada al proyecto CGL2010-1573/BOS, financiado por el Ministerio de Ciencia e Innovación. Los estudios realizados también han sido financiados por el Ministerio de Economía y Competitividad a través del proyecto CGL2013-41642-P/BOS.


"The Clearest Way into the Universe
is through a Forest Wilderness"
John Muir
As

Acknowledgements

This journey has been one of the richest adventures I have ever had.

Javi, from the very start you have supported me to do my PhD in the tropics, which was actually a dream for me. Although we had many unanswered questions in the beginning you showed me that where there is a will, there is a way. And look now where it brought us, my interest in the tropics has only grown exponentially. Thank you for always believing in me, shedding light on issues when I was stuck and for the many good moments we shared in the office and in the field. Apart from being my supervisor, it feels like you have become a friend.

Laura, thank you for being a wonderful person that supported me every second during this fascinating exploration of the virus world. I had no previous experience in virus research and you passed me the interest (virus) and passion you have for this extraordinary symbionts. Who had thought that we would have made such a big jump from papillomaviruses to CRESS DNA viruses, one of the tiniest viruses on earth. From the moments we did not know what we amplified in the lab, through hearing it could be a highly likely artifact, till the final proof we found something really cool, it has been a rollercoaster. Every time I left again for an “estancia”, you wished me the best like I was part of your family.

All the “becarios” from “la planta 9”! Since the moment I first stepped foot at the department, you have made this experience more interesting than ever. Thank you Jasper for being my Flemish speaking buddy and for all your help. Sofia, for all the fun during climbing, the padel and squash tournaments and for your optimism. Irene, for your advice on speaking proper Castellano and accepting my Ecuadorian accent. Sheyla, for your laugh and many hilarious conversations. Anton, for your help in the lab. Joaquin, for the relaxing beer times. Pablo, for our nice conversations. Miche, for flourishing up the department with another exotic accent. Mateja, for all our nice talks and your delicious Gin Tonics. All you guys left the department gradually, and I wished I had spent more time with you. Later, new forces joined the group. Guille, thank you for our great conversations on the tropics while sharing beers. Javi (P), for finally realizing I’m from Belgium and not from France. Amparo, for being one of nicest persons I met and our fun times in Copenhagen and Lund. Bea, for showing me your passion for birding. Alex, for your coolness. Elena, for being the new generation of Batwomen. Richard, for our many “poxy” conversations. Shein, for our shared love for Belgium. Jorge, for all our lab hours together and the great MicroBio Christmass song we wrote together. Maria, for all our fun and “shabby” talks. I think we can finally go celebrate in “Kapital” now!

Many thanks go to all the professors of the department. Especially to Pepe, for your interesting view on the world and mathematical insights. Telle, for your trust and good advice every time I would leave to another remote corner of the world. Thomas, for our funny conversations in the hall. Alvaro,

for asking me how everything's going with my wedding paperwork. Chechu, for taking me on your white stork field expeditions. And many more thanks for all people who helped me along the way.

To all my friends and colleagues at the Museo Nacional de Ciencias Naturales and the Jardin Botánico. Octavio, for being one of my closest friends since we met in Ecuador in 2009. You are one of the most inspiring biologists I ever met. Thank you for all our great times in Spain and abroad and I'm really looking forward to keep on going to the field with you. Vladi, for all the fun we had since we landed in Madrid, you received us in your house several times when we couldn't find a place to stay, and made it even better during our trips through the Madrilean nights. Rigo, for our great times in the center of Madrid talking about haemosporidians and less serious topics and for arranging that the Iberian Lynx crossed our way 10 meters in front of us. Marcos, for all the fun, your spectacular Brazilian food and our most energetic paddle tournaments. I hope to come visit your wonderful country someday. Ramon, for our interesting and mostly absurd conversations. All the other "becarios" which made my stay in Madrid one I will never forget. Finally, a big thank you to Jesus Muñoz, who has been the person who opened the door to all my experiences in tropical Ecuador during the master program. I always think by myself: 'What would my life look like if our paths hadn't crossed?" You were always the person that took care of our "Master" family in Quito.

This thesis is the fruit of many visits and collaborations with national and international researchers. First of all, we established a long term project on avian malaria along the slopes of the Podocarpus National Park in South Ecuador in collaboration with Nikolay Aguirre from the Universidad Nacional De Loja and Elisa Bonaccorso from the Universidad Técnica IndoAmérica. Thank you Nikolay and Elisa, for your support that formed the base of this project and more to come. During the first year of my thesis, I spent 6 months in the amazing cloud forests of Ecuador collecting blood parasites, feather mites and viruses along with local and international students. It was a fantastic time with spectacular birding with many people that helped me in the field. Hector, thanks for our many laughs in the field and your excellent knowledge of tropical birds. Josue, for your enthusiasm and your passion for birds of prey, I'm sure we'll meet again in the future. Anahi, for your energy and eternal laugh, we had fantastic views and birds up there in Cerro Toledo at 3000 m. Thanks "Amiguita" for keeping me company during the first two months. Robin and Melissa, my Belgian friends that flew over especially to have a taste of the Ecuadorian avifauna. Nacho, for your eternal energy and motivation, and the many excellent nights in Loja and San Pedro, you know we are waiting for you in Ecuador! Maru, for all your help holding our favorite hummingbirds: the pufflegs or "cotonetitas".

I had the pleasure to visit Heather Proctor, an expert on the diversity of feather mites worldwide. Thank you Heather for being such an inspiring and nice person and receiving me in your interesting lab. You showed me that feather mites are the most fascinating symbionts of birds, and that so many mysteries still surround them. I promise I will continue studying them! Xi and Zhuang, thanks for our

great trip to Banff and Jasper National Park. Janos, for our fantastic mist-netting every Saturday morning along the North Saskatchewan river, with fresh coffee and Hungarian pastries. Thanks for receiving me like I was your own family and for the great Canadian Thanksgiving dinner! Jeffrey, for the many good conversations and trip to Elk National Park, where we had great Bison encounters.

My next visit was to Gediminas Valkiūnas, the world reference on the morphological identification of blood parasites, during a three month visit at his laboratory in Vilnius. Thank you Gediminas for receiving me so nicely and introducing me to the fascinating world of blood parasite microscopy. Together we analyzed the blood parasites infecting hummingbirds and other neotropical birds, which resulted in chapter 2 of this thesis and the publication of a very nice *Haemoproteus* species of the Rufous capped Sparrow in Ecuador, together with Nubia Matta and other collaborators from the Universidad Nacional de Colombia in Bogota (Mantilla *et al.* 2016). We still have to analyze many blood slides from birds of Ecuador and French Guiana which warrants many more exciting microscopy hours. Mitko and Olena, my office mates in Vilnius, thank you for the many beers and chocolates we shared in the Nature research center and in "Alaus Namai"! You made my stay in Vilnius unforgettable. Vaidas, for our fantastic trip to Rybachi, my first time in Russia to the place where you guys spent so many summers. Hope to take you soon to Ecuador!

Last year I visited Staffan Bensch from the University of Lund, Sweden, during a 2 month stay at his molecular ecology and evolution laboratory. Thanks Staffan, for your warm welcome and our many games of "Innebandy" (Swedish hockey), I almost felt Swedish! Elin, for our many great talks and your fantastic Swedish taco-evening. It meant a lot to me to feel part of your culture. My great office mates Talatu, Julian, Josue, Rosa and Pablo for our 'fika' times and trips to Lund Center.

Finally, we are setting up a long term monitoring project on hummingbird malaria in the Nouragues Research station in French Guiana, in collaboration with Borja Milá and Christophe Thébaud. Thanks Borja for the opportunity to see one of the most wild places on earth in French Guiana. In between the howler monkeys and macaws, we had the chance to study the most fascinating understory birds together. I am really looking forward to go back and to continue learning from your experience, including mist-net "expert furling"! Alvar, thanks for the great moments in French Guiana, we had a fantastic time talking about crazy birds and "Matt Damon", either with a Tipunch or a hummingbird in our hands.

To all my friends from Belgium I could see once in a while, every time it felt very nostalgic to see you guys again. Jonas, Robin, Laurens & Vincent, we still represent the most diverse Bios crew ever, and I sincerely hope to share some time together soon. Jean, even I speak to you once a year, I feel nothing has changed. "Cualquier cosa, me avisas amigo!" All my friends from my hometown Overijse, I am sure our paths will cross again soon.

Of course, nothing of this would have been possible without the continuous support and love of my family, even if I was far away most of the time. Mam, thank you of always believing in me and my dreams, your love goes beyond any distances. Pake, for your passion for nature, and the way you showed me its mysteries from the very beginning. Jess, for being the coolest and most supporting sister ever. I love when you tell everybody that I study malaria in jungle birds, it sounds very adventurous. Falco, for having joined the family 4 years ago, we had great times enjoying good food, drink and music. Oma & Peter, for your continuous support and interest in what I do. Papetje, for being the most inspiring grandfather, and for all the stories we exchanged with a nice “Hoegaarden” in our hands.

Melinda, since we first met in Quito our love & friendship has been growing constantly. You are the most beautiful person I know, thank you for being there for me all the time. I’m looking forward to continue to explore the world with you.

Contents

Abstract

Introduction

Why is there such variation in host specificity? Most symbionts (parasites, mutualists, commensals) are specialist, i.e. they have specialized their lifestyle in one single host species, while others are generalist interacting with many host species. In this thesis, we aim to identify historical and ecological determinants of host specificity in avian blood parasites and viruses in Neotropical birds. The tropics provide an ideal scenario to explore the relationship between host specificity and host diversity, as the latter could govern the evolution of host specificity through dilution and amplification effects. At the ecosystem scale host range is also determined by the coevolutionary history of hosts and symbionts, where strict cospeciation maintains specialization, and host-switching promotes the evolution of generalist symbionts. We will study the influence of these historical processes on the evolution of host specificity of avian blood parasites, avipoxviruses and CRESS (circular rep-encoding single stranded) DNA viruses. At the interspecific scale we will focus on hummingbirds, whose particular blood features offer a unique niche for blood parasites. The concept of ecological specialization of symbionts is equivalent to niche specialization of free living species, and is crucial to understand the architecture of host parasite networks and how species diversification takes place. Since we worked in underexplored tropical areas, the thesis is also focused towards biodiscovery, which is presented by the characterization of new blood parasite species, *Avipoxvirus* strains and CRESS DNA viruses. Taken together, this comprehensive analysis of symbiont host specificity will contribute to the discovery of new species and symbiotic lifestyles, giving an excellent opportunity to improve our understanding on the evolution of host-symbiont interactions in megadiverse environments.

Objectives and Results

The general objective of this thesis is to make a significant contribution to the knowledge of ecology and evolution of host-symbiont relationships by analyzing the factors that promote or compromise specialization versus generalization in symbiotic interactions.

Evolution of host specificity. Where bird diversity is one of the highest worldwide, high host specificity may be penalized by the reduction of relative host availability, causing a decrease in parasite transmission, which is known as the dilution effect. In this scenario natural selection could favor the evolution of generalist parasites, which would benefit from an increased encounter rate with adequate hosts, properly known as the amplification effect. In **chapter 1** we analyze the host specificity of *Haemoproteus* and *Plasmodium* communities in a megadiverse area in South Ecuador and compare it to multiple other parasite communities across the globe in order to know if these parasite communities are more generalist than in temperate areas. We also explored how the parasite communities evolved in this biodiversity hotspot, which could have been the result of local speciation events or multiple colonization events involving host switching. We found high levels of host generalization for both parasite genera, and the mean host range of the *Haemoproteus* community in Ecuador was significantly higher than other parasite communities in other areas outside the Neotropics. Generalist *Haemoproteus* parasites in this bird community had diverse phylogenetic ancestry, were closely related to specialist parasites and were apparently endemic to the Amazon showing that different parasites have independently evolved into host generalists in this region. Finally, we show that *Haemoproteus* communities in Ecuador and South America are more generalist than in temperate areas, making this continent a hotspot of generalist *Haemoproteus* parasites for wild birds.

Host specialization in a unique habitat. The evolutionary and ecological processes involved in niche filling are still a major puzzle in biology. When new niches become available for parasites they can provide a unique opportunity to specialize in a new life form or they can be occupied by generalist parasites broadening their niches through host switching by ecological fitting. By studying special environments for parasites we can gain insight how host specificity evolves. In **chapter 2** we take a closer look to the blood parasites of hummingbirds. Hummingbirds possess a unique physiology in the vertebrate world and their blood holds special attributes such as small erythrocyte size and high erythrocyte concentrations compared to the great majority of bird diversity. We set out to explore if (1) the community of *Haemoproteus* parasites of hummingbirds is the result of single or multiple colonization events, (2) to what extent these parasites are specialized in hummingbirds or shared with other birds, and (3) how hummingbirds contribute to sustain the populations of these parasites, in terms of both prevalence and infection intensity. Hummingbirds hosted a phylogenetically diverse assemblage of generalist *Haemoproteus* lineages shared with other host orders, indicating multiple colonization events. Among these parasites, *Haemoproteus witti* stood out as the most generalist. Interestingly, we found that infection intensities of this parasite were extremely low in passerines (with no detectable gametocytes) but very high in hummingbirds, with many gametocytes seen. Moreover, infection intensities of *H. witti* were positively correlated with prevalence across host species.

The nature of emerging disease. The emergence of wildlife diseases depends on a combination of factors such as the coevolutionary history of hosts and pathogens, climate, or host community composition. Most research has focused on understanding emerging infectious diseases when they already cause trouble to biodiversity, but fewer studies have explored how pathogens circulate in natural populations before they provoke an outbreak. A profound problem with the understanding of emerging diseases is that we seldom know about the background biology of emergent pathogens, because the interest to study them arises after they emerged. From this perspective, studying the diversity and prevalence of circulating pathogens and the evolutionary relationships between these and their hosts is a critical issue. In **chapter 3** we will explore to what extent wild bird populations are infected with *Avipoxvirus* strains in two megadiverse environments of Ecuador and French Guiana. We will analyze the host specificity and distribution of all known *Avipoxvirus* strains worldwide to gain knowledge on how *Avipoxvirus* circulates in wild bird communities and what makes these viruses especially prone to cause disease emergence. Our study at the community level shows that distantly related *Avipoxvirus* strains circulate at very low prevalence in continental tropical South America. An analysis of the host specificity and geographic distribution of all *Avipoxvirus* strains known worldwide finds that these viruses are usually host generalists (particularly those in the fowlpox clade), and are interchanged on a global scale. When *Avipoxvirus* assemblages are composed of generalist strains with different ancestry and widespread distribution, this combination of characters may make these typically scarce viruses perfect candidates to emerge under favorable ecological conditions.

Biodiscovery. Understanding how living organisms evolved in such an astounding diversity remains a fundamental scientific challenge which is critical to design conservation programs to protect biodiversity for future generations. We worked in multiple areas in the Neotropics where no research has been carried out on these symbionts and only a small part of the diversity of symbionts has been described so far. In **chapter 4** we present the characterization of two new CRESS DNA viruses isolated from a cutaneous lesion, caused by *Avipoxvirus*, in a blue and gray tanager (*Thraupis episcopus*) of Southern Ecuador. Both viruses present a replication-associated protein (Rep) and one to two open reading frames (ORF) which represent a putative Cap protein. Within these ORFS corresponding to the capsid proteins, we detected intrinsically disordered regions (IDR), putative protein binding regions, several motifs related to rolling circle replication and nuclear localization signals (NLS), providing further evidence on the function of both proteins. We describe a new characteristic IDR profile for CRESS DNA viruses. Both viruses show low similarity between each other (<60%) and with other known CRESS DNA viruses. By analyzing evolutionary relationships within the CRESS DNA virus diversity, we show that host switching has been important during their evolutionary history.

Conclusions and Relevance

I. The evolution of generalist parasites has been promoted multiple times during the evolutionary history of *Plasmodium* and *Haemoproteus* in a megadiverse bird community in Ecuador. Moreover, the mean host specificity of the local *Haemoproteus* community was significantly higher than others outside the Neotropics. *Haemoproteus* parasite assemblages in Ecuador and South America are more generalist than elsewhere, making this continent a hotspot of generalist *Haemoproteus* parasites of wild birds.

II. In a tropical bird community in Ecuador, the generalist *Haemoproteus* parasites have a wide phylogenetic ancestry, they are closely related to specialist parasites and they are endemic to the Amazon forest, suggesting they evolved towards generalists upon colonization of this megadiverse region.

III. Hummingbirds are colonized by generalist *Haemoproteus* parasites which are all shared with other host orders which supports the idea that the unique blood physiology has not promoted parasite specialization (but see **V**). Instead this unique niche has been occupied by parasites which generally spill over from other sympatric bird species.

IV. Andean hummingbirds are infected with blood parasites belonging to various *Haemoproteus* clades with wide phylogenetic origins within the worldwide *Haemoproteus* diversity. This finding supports that multiple colonization events by *Haemoproteus* parasites have affected hummingbirds during their evolutionary history and shows that host-switching has been an important process in shaping the diversity of parasites in this unique family of birds.

V. *Haemoproteus witti* is a super generalist parasite infecting hummingbirds and other bird orders, but is more dependent on hummingbirds both in terms of prevalence and gametocymia compared to other bird species. This unveils hummingbirds as important reservoirs for this parasite in this region. The distinction in reservoir importance challenges the way we interpret host specialization in blood parasites and other host parasite systems, and requires the use of multiple parasite detection techniques to reveal true patterns of host specificity.

VI. Bird communities in Ecuador and French Guiana show an extremely low prevalence and diversity of *Avipoxvirus* lesions affecting local bird species. Two new strains were found at different altitudes along an elevational gradient in Ecuador while no strains were detected in the lowland Amazon of French Guiana. Both strains have distant evolutionary trajectories with one strain placed in an American clade of Axipoxviruses while the second one is closely related to a strain found in the Madeira Archipelago. These results reflect the low circulation and diversity of *Avipoxvirus* lesions in tropical bird communities, and require future investigations to confirm this as a global phenomenon.

VII. Avipoxviruses are usually very generalist and they are widely distributed over continents. These two attributes help us to understand why avipoxviruses are good candidates to emerge under favorable ecological and evolutionary conditions, as has occurred multiple times in the past.

VIII. Two novel CRESS DNA viruses are described on an *Avipoxvirus* lesion of a common bird species in Ecuador. This discovery expands the current knowledge of these viruses about their potential hosts, distributions and molecular organization. Extensive host switching has taken place during the evolutionary history of these viruses between hosts and habitats of with very diverse origins.

Resumen

Introducción

¿Por qué hay tanta variación en la especificidad? La mayoría de los simbiontes (parásitos, mutualistas, comensales) son especialistas, i.e. han especializado su estilo de vida en una especie hospedadora, mientras otros son muy generalistas interactuando con muchas especies hospedadoras. En la presente tesis, queremos identificar determinantes históricos y ecológicos de la especificidad de parásitos sanguíneos y virus en aves Neotropicales. Los trópicos proveen un escenario ideal para explorar las relaciones entre especificidad y la diversidad de hospedadores, ya que lo último podría gobernar la evolución de la especificidad a través de los efectos de dilución e amplificación. A escala ecosistémica la especificidad también está determinada por la historia coevolutiva de hospedadores y simbiontes, ya que la coespeciación mantiene la especialización, y los cambios de hospedadores promueven la evolución de simbiontes generalistas. Estudiaremos el efecto de estos procesos históricos en la evolución de la especificidad de parásitos sanguíneos, *Avipoxvirus* y CRESS (circular rep-encoding single stranded) DNA virus. A escala interespecífica nos enfocaremos en los colibríes, cuyos parámetros sanguíneos ofrecen un nicho único para los parásitos sanguíneos. El concepto de la especialización ecológica es equivalente a la especialización en el nicho ecológico para las especies en general, y es importante para entender la arquitectura de las redes hospedador-parasito y cómo ocurre la diversificación. Como hemos trabajado en áreas neotropicales poco exploradas, una gran parte de la tesis esta también dedicada al biodescubrimiento, lo cual está presentado por la caracterización de nuevas especies de parásitos sanguíneos, cepas de *Avipoxvirus*, y genomas de CRESS DNA virus. En general, los análisis de la especificidad de los simbiontes aportarán al descubrimiento de nuevas especies e interacciones simbióticas, dando una oportunidad excelente para mejorar nuestro conocimiento sobre la evolución de relaciones hospedadoras-parásitos en ambientes megadiversos.

Objetivos y resultados

El objetivo general de la tesis es hacer una contribución significativa al conocimiento de la ecología y la evolución de las relaciones huésped-simbionte mediante el análisis de los factores que promueven o limitan la especialización frente a la generalización en las interacciones simbióticas.

Evolución de la especificidad. Donde la diversidad de aves es la más alta globalmente, la especialización puede ser perjudicada por la reducción en la abundancia relativa de hospedadores, causando una disminución en transmisión, conocido como el efecto de dilución. En estas circunstancias la selección natural podría promover la evolución de parásitos generalistas, los cuales se beneficiarían de un incremento de la tasa de encuentro con hospedadores adecuados, conocido como el efecto de ampliación. En el **capítulo 1** se analiza la especificidad de las comunidades de

Haemoproteus y *Plasmodium* en una zona megadiversa en el sur de Ecuador para compararlas a múltiples comunidades de parásitos en todo el mundo con el fin de saber si estas comunidades de parásitos son más generalistas que en las zonas templadas. También exploramos cómo las comunidades de parásitos evolucionaron en este hotspot de biodiversidad, lo que podría haber sido el resultado de eventos de especiación locales o varios eventos de colonización por cambio de hospedadores. Se encontraron altos niveles de generalización para los dos géneros de parásitos, y la especificidad promedia de la comunidad *Haemoproteus* en Ecuador fue significativamente más alto que en comunidades de parásitos en otras áreas fuera del Neotrópico. Los *Haemoproteus* generalistas en esta comunidad de aves tenían diversos orígenes filogenéticos, eran muy relacionados con parásitos especialistas y eran aparentemente endémicos de la Amazonía lo cual sugiere que los diferentes parásitos han evolucionado independientemente hacia generalistas en esta región. Finalmente se muestra que las comunidades de *Haemoproteus* en Ecuador y América del Sur son más generalistas que en las zonas templadas, por lo cual este continente forma un reservorio de parásitos de *Haemoproteus* generalistas para las aves silvestres.

Especialización en un hábitat único. Los procesos evolutivos y ecológicos que intervienen en el llenado de nichos siguen siendo unos de los problemas importantes en la biología. Cuando aparecen nuevos nichos disponibles estos pueden proporcionar una oportunidad única para los parásitos para especializarse en una nueva forma de vida o pueden ser colonizados por parásitos generalistas ampliando sus nichos por medio de encaje ecológico. A través del estudio de ambientes especiales para los parásitos podemos conocer mejor cómo evoluciona la especificidad. En el **capítulo 2** nos enfocamos en los parásitos sanguíneos de los colibríes. Estas aves poseen una fisiología única en el mundo de los vertebrados y su sangre contiene atributos especiales tales como el pequeño tamaño de los eritrocitos y las altas concentraciones de eritrocitos en comparación con la gran mayoría de la diversidad de aves. Proponemos analizar si (1) la comunidad de parásitos de *Haemoproteus* de los colibríes es el resultado de uno o varios eventos de colonización, (2) en qué medida estos parásitos están especializados en colibríes o son compartidos con otras aves, y (3) cómo contribuirán los colibríes al sostenimiento de las poblaciones de los parásitos *Haemoproteus*, tanto en términos de prevalencia e intensidad de infección. Demostramos que los colibríes tienen un conjunto de *Haemoproteus* generalistas con orígenes filogenéticos diversos y que comparten estos parásitos con otros órdenes de aves, lo que indica que múltiples eventos de colonización han ocurrido en la historia evolutiva de esta comunidad de parásitos. Entre estos parásitos, *Haemoproteus witti* se destacó como el parasito más generalista. Curiosamente, encontramos que las intensidades de infección de este parásito eran extremadamente bajos en paseriformes (sin gametocitos detectables) pero muy altas en colibríes, con la detección de muchos gametocitos. Además, la intensidad de la infección de *H. witti* se correlacionó positivamente con la prevalencia a lo largo de todas las especies de hospedador infectadas.

La naturaleza de las enfermedades emergentes. La aparición de enfermedades infecciosas depende de una combinación de factores tales como la historia evolutiva de los hospedadores y los patógenos, las condiciones climáticas, o la composición de la comunidad de hospedadores. La gran mayoría de la investigación se ha centrado en entender las enfermedades infecciosas emergentes cuando ya han surgido como problemas para la biodiversidad, pero pocos estudios han explorado a qué nivel los patógenos circulan en poblaciones naturales antes de provocar un brote. Otro problema con el entendimiento de las enfermedades emergentes es que algunas veces no conocemos la biología básica de patógenos emergentes, debido a que el interés para el estudio de las mismas surge en el momento que forma un problema. Desde este punto de vista, el estudio de la diversidad y la prevalencia de patógenos circulantes y las relaciones evolutivas entre estos y sus hospedadores es un tema sumamente importante. En el **capítulo 3** exploraremos en qué medida las poblaciones de aves silvestres están infectados con cepas de viruela aviar en dos ambientes megadiversos del Ecuador y la Guayana Francesa. Analizaremos la especificidad y la distribución de todas las cepas de viruela aviar conocidas en el mundo para obtener conocimientos sobre cómo *Avipoxvirus* circula en comunidades de aves silvestres y que hace estos virus especialmente propensos a causar la aparición de un brote. Nuestro estudio a nivel comunitario muestra que las cepas de viruela aviar son filogenéticamente diversas y sugiere que circulan con una prevalencia muy baja en el trópico de América del Sur continental. Un análisis de la especificidad y la distribución geográfica de todas las cepas de viruela aviar conocidas indica que estos virus suelen ser generalistas (particularmente aquellos en el clado de fowlpox), y que se intercambian a escala global. Cuando las comunidades de *Avipoxvirus* se componen de cepas generalistas con diferente ascendencia filogenética y una distribución generalizada, esta combinación de caracteres puede hacer que estos escasos virus sean candidatos perfectos para surgir en condiciones ecológicas favorables.

Biodescubrimiento. La comprensión de cómo los organismos vivos evolucionaron en una diversidad tan asombrosa sigue siendo un reto científico fundamental, que es importante para diseñar programas de conservación para proteger la biodiversidad para las futuras generaciones. Hemos trabajado en múltiples áreas en el Neotrópico, donde se ha desarrollado poca investigación en estos simbiontes y donde sólo se ha descrito una pequeña parte de su diversidad hasta ahora. En el **capítulo 4** se presenta la caracterización de dos nuevos CRESS DNA virus aislados de una lesión cutánea, causada por *Avipoxvirus*, en una tangara azuleja (*Thraupis episcopus*) en el Sur de Ecuador. Ambos virus presentan una proteína de replicación (Rep) y uno a dos marcos de lectura abiertos (ORF) que representan una posible proteína de la cápsida (Cap). Dentro de los ORFS correspondientes a las cápsidas, se encontraron regiones intrínsecamente desordenadas (IDR), regiones putativas de unión a proteínas, varios motivos relacionados con la replicación por círculo rodante y señales localización nuclear (NLS), proporcionando más evidencias de la funcionalidad de ambas proteínas. Se describe un nuevo perfil IDR característico de los CRESS DNA virus. Ambos virus muestran una baja

similitud entre sí (<60%) y con otros CRESS DNA virus. Virus muy relacionados fueron aislados de huéspedes de origines muy diversos, demostrado que cambios de hospedadores han sido muy importantes durante su historia evolutiva.

Conclusiones y Aportaciones científicas

I. La evolución de los parásitos generalistas se ha promovido varias veces en la historia evolutiva de *Plasmodium* y *Haemoproteu*s en una comunidad de aves megadiversa en Ecuador. Además, la especificidad media de la comunidad local *Haemoproteus* fue significativamente más alta que en otras comunidades fuera del Neotrópico. Las comunidades de *Haemoproteus* en Ecuador y América del Sur son más generalistas que en otros lugares, lo cual hace este continente un reservorio de parásitos *Haemoproteus* generalistas en aves silvestres.

II. En una comunidad de aves tropicales en Ecuador, los parásitos *Haemoproteus* generalistas tienen una amplia diversidad filogenética, están relacionadas con parásitos especialistas y son endémicas de la selva amazónica, lo que sugiere que evolucionaron hacia generalistas cuando colonizaron esta región megadiversa.

III. Los colibríes han sido colonizados por parásitos *Haemoproteus* generalistas que son todos compartidos con otros órdenes de aves lo cual apoya la idea de que la fisiología única de la sangre no ha promovido la especialización de los parásitos (pero véase **V**), pero ha sido ocupada por parásitos que generalmente se transmiten desde otras especies de aves simpátricas.

IV. Los colibríes andinos están infectados por parásitos sanguíneos pertenecientes a varios clados de *Haemoproteus* con orígenes filogenéticos amplios dentro de la diversidad mundial de *Haemoproteus*. Este hallazgo apoya a que múltiples eventos de colonización por parásitos de *Haemoproteus* han afectado a los colibríes durante su historia evolutiva y demuestra que el cambio de hospedador ha sido un proceso importante durante la evolución de la diversidad de parásitos en esta familia única de aves.

V. *Haemoproteus witti* es un parasito muy generalista que infecta colibríes y otros ordenes de aves, pero surge como más dependiente de colibríes tanto en términos de prevalencia y gametocitos, comparado a otras aves. Esto implica que colibríes son reservorios importantes de este parasito en esta región. La diferencia en la importancia como reservorio cuestiona la manera de interpretar la especialización en parásitos sanguíneos y en otros grupos de parásitos, y requiere el uso de múltiples técnicas de detección para revelar los verdaderos patrones de especificidad.

VI. Varias comunidades de aves en Ecuador y Guayana Francesa tienen una prevalencia y diversidad muy baja de lesiones de viruela aviar que afectan a las especies de aves locales. Dos nuevas cepas que afectan principalmente a las tangaras fueron encontradas a diferentes altitudes a lo

largo de un gradiente altitudinal en Ecuador, mientras que no se detectó ninguna cepa en las tierras bajas del Amazonas de la Guayana Francesa. Ambas cepas tienen trayectorias evolutivas muy divergentes, en lo cual una cepa está emparentado con *Avipoxvirus* del nuevo mundo mientras que la segunda está estrechamente relacionada con una cepa que se encontró en el archipiélago de Madeira. Estos resultados reflejan la baja circulación y diversidad de las lesiones de viruela aviar en las comunidades de aves neotropicales, y requieren futuras investigaciones para confirmar estas observaciones como fenómeno universal.

VII. *Avipoxvirus* son habitualmente muy generalistas y que se distribuyen ampliamente sobre los continentes. Estos dos atributos ayudan a entender por qué las cepas de *Avipoxvirus* son buenos candidatos para emerger en condiciones ecológicas y evolutivas favorables, como ha ocurrido varias veces en el pasado.

VIII. Dos nuevos CRESS DNA virus están descritos provenientes de una lesión cutánea de *Avipoxvirus*. Este descubrimiento amplía el conocimiento actual de estos virus sobre sus posibles anfitriones, distribución geográfica y organización molecular. Cambios de hospedadores han gobernado la historia evolutiva de estos virus entre huéspedes y hábitats de orígenes muy diversos.

General introduction

Host specificity is one of the most important characteristics of a symbiont and relates to the concept of the ecological niche of free-living organisms (Schmid-Hempel 2011). A specialist is a symbiont exploiting one host species whereas a generalist infects multiple host species. On macro evolutionary scale, generalist symbionts can evolve by regular host switching or as a result of multiple speciation events in the host species without speciation in the symbiont species (Poulin 2006). On micro evolutionary scale, host specificity is determined by opportunities for colonization and the availability of suitable host species (Poulin 2006). Natural selection acts on the evolution of host specificity in no fixed direction: it mainly depends on opportunities of host switching, availability of suitable hosts and on how host switching affects symbiont fitness (Poulin 2006). The great majority of symbionts (parasites, mutualists and commensalists) are specialists, interacting with one host species, while a smaller percentage consists of generalists (Poulin & Keeney 2008). This generates an outstanding research question: "Why is there such variation in host specificity?".

In theory, all symbionts could benefit of the exploitation of multiple hosts available in their range (Hellgren, Pérez-Tris & Bensch 2009). Hence, one can ask why most symbionts not exploit every ecologically or phylogenetically similar host available around them. The network of host-symbiont interactions is structured on the ecological level, through nestedness and modularity patterns (Montoya, Pimm & Solé 2006) and on an evolutionary level through co-speciation and host-switching during the coevolution of hosts and symbionts (Hoberg & Brooks 2008). Moreover, all symbiotic relationships take place in an explicit geographical context, which can promote or restrict the formation of new interactions throughout different host populations (Thompson 2005). Therefore, the knowledge of which factors promote or compromise specialization of generalization of symbiotic relationships is vital to understand their ecology and evolution.

The trade-offs between specialization and generalization, dilution and amplification effects

The fitness of each symbiont depends greatly on its transmission success, but not all potential hosts are as compatible to one symbiont, as some are easily colonized and present a suitable habitat for reproduction (Johnson *et al.* 2013a). Other hosts can be of difficult access or pose trouble to the reproduction of the symbiont once colonized, through resistance mechanisms or host mortality (Combes 1997). Therefore, the local success of a symbiont depends heavily on the proportion of competent hosts in the population: when this proportion grows, an amplification effect occurs resulting in an increase of transmission of the symbiont. In contrast, when this proportion decreases, a dilution effect takes places reducing the prevalence of a symbiont, which could eventually lead to its local extinction (Keesing *et al.* 2010). In this scenario, natural selection can favor specialization if the symbiont obtains minimal contact with incompatible hosts. Nevertheless, specialization often implies losing mechanisms to invade and exploit alternative hosts (Garamszegi 2006), and if the local

abundance of the main host decreases under a certain threshold, the process of transmission risks a collapse (Rudolf & Antonovics 2005). These processes may compromise symbiont transmission at places where host diversity is high, since an increase in local diversity normally implies a decrease in the relative host abundance of coexisting species (Ostfeld & Keesing 2012). Indeed the dilution effect is known as the negative relationship between local host diversity and infection risk (Keesing, Holt & Ostfeld 2006).

If the dilution effect poses a selective pressure on the symbiont host specificity towards and increased host range where host diversity is higher, we could expect that the evolution of host-symbiont interactions produces spatial-temporal variation in host specificity levels. For example, the host range of a symbiont can be restricted through phylogenetic constraints if host-switching cannot widen the interaction with other host species (Ricklefs, Fallon & Bermingham 2004; Poulin & Keeney 2008). Competition between symbionts can also promote specialization through niche partitioning (Schluter 2000; de Roode *et al.* 2005). Moreover, it is predicted that specialization evolves when the benefits of generalization (through amplification effects) do not overcome its costs, which are related to the diverse mechanisms of exploitation and evasion of a wide diversity of host immune systems. This idea is known as the popular saying: "Jack-of-all-trades, master of none" (Hellgren *et al.* 2009).

Diversity and host specificity at different scales

The concepts of amplification and dilution effects have been central in epidemiology. Its theoretical framework (Keesing *et al.* 2006) and its practical application (Johnson *et al.* 2013a; b) have contributed to our understanding of the value of ecosystem services of biodiversity. Host diversity defines the context in which evolutionary history and adaptation drive the specialization of host-symbiont interactions. These processes operate on different scales which we will explore in this thesis: between ecological communities and between different species in a community (Ostfeld & Keesing 2012). In the Neotropics, where bird diversity is one of the highest worldwide (Myers et al. 2000), high host specificity may be penalized by the reduction of relative host availability, causing a decrease in parasite transmission. In this scenario natural selection could favor the evolution of generalist parasites, which would benefit from an increased encounter rate with adequate hosts. We can thus expect that megadiverse areas have more host generalist species due to increased colonization opportunities and more available host species.

Historical and ecological processes acting on host specificity

Hosts and symbionts need to have geographical overlap in their ranges in order to get in contact, which depends on historical processes and ecological determinants of these distributions (Hoberg & Brooks 2008; Pérez-Rodríguez *et al.* 2013a; b). Historical processes determine host specificity on a global scale: there are symbionts exclusive for one host species (Pérez-Tris *et al.* 2007) while others

are capable to exploit many host species throughout their geographical range (Ewen *et al.* 2012). These differences reflect opportunities and restrictions undergone by the symbionts during their coevolutionary histories with their hosts. Co-speciation maintains host specialization while regular host-switching promotes the evolution of generalist symbionts through ecological fitting (Ricklefs *et al.* 2014b; Araujo *et al.* 2015).

We can thus expect that a host-symbiont relationship is established through two main processes. First, the evolutionary history of hosts and symbionts can condition the actual structure of the interactions on a local scale, through restrictions imposed by co-speciation and host switching opportunities (Ricklefs *et al.* 2014b). Second, the ecology of hosts and symbionts can promote or restrict access from the symbionts to different host species (Poulin 2006; Fuller *et al.* 2012). In order to explore these phenomena, it is useful to work with representatives of an adaptive radiation (a diverse but phylogenetically restricted group (Schluter 2000)), which facilitates the analyses of the relationships between species characteristics and the resulting host-symbiont interactions. The evolutionary and ecological processes involved in niche filling is still a major puzzle in biology (Ricklefs 2010). When new niches become available for parasites they can provide a unique opportunity to specialize in a new life form or they can be occupied by generalist parasites broadening their niches through ecological fitting (Araujo et al. 2015). This can be studied on the ecosystem scale studying different host communities or at an interspecific scale, focusing on different host species.

Emerging disease and host specificity

The degree of host specificity affects the likelihood that a symbiont can switch to a new host species, which may lead to a potential emerging disease (Daszak, Cunningham & Hyatt 2000; Cleaveland, Laurenson & Taylor 2001). The emergence of wildlife diseases depends on a combination of factors such as the coevolutionary history of hosts and pathogens, climate, or host community composition (Jones et al. 2008; Keesing et al. 2010). Most research has focused on understanding emerging infectious diseases when they already cause trouble to biodiversity, but fewer studies have explored how pathogens circulate in natural populations before they provoke an outbreak. A deeper problem with the understanding of emerging diseases is that we seldom know the background biology of emergent pathogens, because the interest to study them arises after they emerged. From this perspective, studying the diversity and prevalence of circulating pathogens and the evolutionary relationships between these and their hosts is a critical issue (Fuller et al. 2012).

Objectives

The general objective of this thesis is to make a significant contribution to the knowledge of ecology and evolution of host-symbiont relationships by analyzing the factors that promote or compromise specialization versus generalization in symbiotic interactions. To this end, we propose a question driven research developed on the community level by exploring host specificity, diversity and prevalence of bird symbionts in multiple communities in the Neotropics and on an interspecific level by studying parasite host specialization in hummingbirds.

Evolution of host specificity

In **chapter 1** we analyze the host specificity of *Haemoproteus* and *Plasmodium* communities in a megadiverse area in South Ecuador and compare it to multiple other parasite communities across the globe in order to know if these parasite communities are more generalist than in temperate areas. We also explored how the parasite communities evolved in this biodiversity hotspot, which could have been the result of local speciation events or multiple colonization events involving host switching. By these means we gain insight into how host specificity evolves in megadiverse environments.

Host specialization in unique habitats

In **chapter 2** we take a closer look to the blood parasites of hummingbirds. Hummingbirds possess a unique physiology in the vertebrate world (Suarez *et al.* 1991) and their blood has special attributes such as small erythrocyte size and high erythrocyte concentrations compared to the great majority of bird diversity (Opazo, Soto-Gamboa & Fernandez 2005; Glomski & Pica 2011). We set out to explore if (1) the community of *Haemoproteus* parasites of hummingbirds is the result of single or multiple colonization events, (2) to what extent these parasites are specialized in hummingbirds or shared with other birds, and (3) how hummingbirds contribute to sustain the populations of these parasites, in terms of both prevalence and infection intensity. This chapter sheds light on the role of niche diversity on the evolution of host specificity in avian blood parasites. It also challenges the current view we have on host specialization, how it is correctly measured which has important implications for understanding disease dynamics.

The nature of emerging diseases

In **chapter 3** we will explore to what extent wild bird populations are infected with *Avipoxvirus* strains in two megadiverse environments of Ecuador and French Guiana. Previous research on *Avipoxvirus* has mainly targeted cases where particular bird species are heavily affected by these virus infections, yet few data are available on what extent these viruses circulate in wild bird communities before their emergence as an infectious disease. Moreover, it is important to determine key characteristics of these viruses which make them especially prone to develop into an emerging disease

when the right conditions for outbreak occur. We will analyze host specificity and distribution of all known *Avipoxvirus* strains worldwide to gain knowledge on how *Avipoxvirus* circulates in wild bird communities and what makes these viruses especially prone to cause disease emergence.

Biodiscovery

The thesis also contains a big biodiscovery part in all four chapters. Understanding how living organisms evolved in such an astounding diversity remains a fundamental scientific challenge which is critical to design conservation programs to protect biodiversity for future generations. We worked in multiple areas in the Neotropics where no research has been carried out on these symbionts and only a small part of the diversity of symbionts has been described so far. In **chapter 4** we characterize two new virus genomes which are associated with an *Avipoxvirus* infection. By studying the presence of *Avipoxvirus* and *Papillomavirus* we suddenly discovered a new diversity of viruses, belonging to circular replication encoding single stranded (CRESS) DNA viruses. We aim to gain insights on the structural and phylogenetic diversity of this megadiverse group of viruses and on how important host switching has been during their evolutionary history

Methodology

Study models

Avian blood parasites

The avian blood parasites studied in this thesis cause avian malaria and similar diseases in birds, and are excellent models to explore the ecology and evolution of host parasite relationships due to their spectacular diversity, cosmopolitan distribution and their facility to sample (Peréz-Tris *et al.* 2005; Bensch, Hellgren & Peréz-Tris 2009). The avian malaria parasites (*sensu lato*) belong to the genera *Plasmodium* and *Haemoproteus* (phylum Protozoa, order Apicomplexa) (Valkiūnas 2005; Peréz-Tris *et al.* 2005). These parasites share characteristics of development related to their sexual and asexual lifecycle, which require an arthropod host (mosquito, biting midge, fly) and a vertebrate host (bird) respectively (Valkiūnas 2005). The primary vectors of *Haemoproteus* are biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) and louse-flies (Diptera: Hippoboscidae) and more than 60 species of culicine and anopheline mosquitoes are known to be competent hosts of a variety of *Plasmodium* species from avian hosts (Valkiūnas 2005). *Plasmodium* and *Haemoproteus* parasites differ in the asexual phase of reproduction (merogony), which also occur in the peripheral blood cells (erythrocytes) of the bird host in *Plasmodium* while in *Haemoproteus* this exclusively happens within the birds' tissues and organs (Valkiūnas 2005). More than 200 morphospecies have been described (Valkiūnas 2005) and more than 2000 lineages have been detected in approximately 1200 bird species (Bensch *et al.* 2009). Lineages are characterized by a unique part of the cytochrome *b* gene that is used as a "barcode" for species determination among the three haemosporidian genera, which has facilitated the use of haemosporidian as models to study the evolutionary ecology of parasites (Bensch *et al.* 2009). These parasites show a huge variation in host specificity, as has been shown by multiple studies (Beadell *et al.* 2004; Križanauskienė *et al.* 2006; Hellgren *et al.* 2009), still the factors driving these differences are poorly understood. Loiseau *et al.* 2012 found that the degree of host specialization was associated to habitat type and host geographical range. Another study found no difference in host specificity of *Haemoproteus* and *Plasmodium* between a temperate forest in Missouri and a tropical rainforest in Ecuador (Svensson-Coelho *et al.* 2014). Geographical barriers can also affect the distribution of generalist or specialist parasites like the strait of Gibraltar in Spain which is an effective barrier for specialist blood parasites (Mata *et al.* 2015). All these examples show that habitat characteristics are important to understand the variation of host specificity in blood parasites (Sehgal 2015).

Avipoxvirus

Avipoxvirus is a serious concern for endemic bird species on islands and in naïve bird populations on the continent (Wikelski *et al.* 2004; van Riper III & Forrester 2007; Lachish *et al.* 2012). The virus

causes the disease known as avian pox and consists of a linear double stranded DNA genome of up to 300 kb and is mainly transmitted through mosquito bites, aerosols and host body contact (Tulman *et al.* 2004; Jarmin *et al.* 2006). The disease is apparent as epidermal nodular lesions on and around featherless areas which is referred to as the cutaneous form (Fig. 1), while the less common diphtheritic form affects respiratory and digestive systems impairing breathing and swallowing (van Riper III & Forrester 2007; Offerman *et al.* 2013). The cutaneous form is the most common among bird species worldwide and could impede sight, feeding or mobility under certain circumstances (van Riper III & Forrester 2007). More than 60 unique strains have been identified in more than 278 bird species (Gyuranecz *et al.* 2013) and 10 virus species have been recognized by the International Committee on Taxonomy of Virus by early 2015 (King *et al.* 2012) (http://www.ictvonline.org/). The study of host specificity in viruses is extremely important as it helps to explore why host shifts occur, which could lead to possible pandemics (Gao *et al.* 1999; Webby & Webster 2001; Longdon *et al.* 2014). There is a huge variation in host specificity between different *Avipoxvirus* strains, where canarypox strains seem to be more specialized in passerines while fowlpox strains infect hosts of different orders. Still, no specific analyses have been performed to study if there is a considerable difference in host specificity of *Avipoxvirus* isolates in the two main clades.

Figure 1: Detail of *Avipoxvirus* lesions caused by the two new strains found in this thesis, from the blue-gray tanager *Thraupis episcopus* (left) and the blue-capped tanager *Thraupis cyanocephala* (right).

CRESS DNA viruses

Circular rep-encoding single-stranded (CRESS) DNA viruses represent the smallest known pathogens infecting eukaryotic organisms (Rosario, Duffy & Breitbart 2012). They include plant pathogens of the family *Geminiviridae* and *Nanoviridae* and animal viruses belonging to the families *Anelloviridae*, *Parvoviridae* and *Circoviridae* (King *et al.* 2012). The family *Circoviridae* is of particular interest in

birds since they have been linked to immunosuppressive threats to avian species and beak and feather disease (Todd 2000, 2004; Johne *et al.* 2004; Jackson *et al.* 2015). Moreover, it has been shown they are associated with growth delay and developmental abnormalities (Stewart, Perry & Raidal 2006). They typically consist of a circular genome, presenting two major open reading frames (ORFs) which code for a capsid protein (Cap) and a replication protein (Rep), separated by a conserved nomamere motif (5'-NANTATTAC-3') in between the 5'-ends of both ORF's (Rosario *et al.* 2012). They evolve rapidly due to high mutation and recombination rates (Duffy, Shackelton & Holmes 2008; Lefeuvre *et al.* 2009), which make them good candidates to make host shifts and or develop into a disease causing concerns in the conservation of wildlife (Rosario *et al.* 2012). The fecal-oral route of transmission is likely, but vertical transmission has been reported in some cases for *Circovirus* (Todd 2004). Most of known circoviruses are pathogens, which cause immune suppression and damage in the lympho-reticular tissues, but the pathogenesis of many CRESS DNA virus is unknown.

Neotropical birds

The Neotropics span from Central America to South America and contain about 3300 bird species, which make it the most diverse bioregion on the planet in terms of bird diversity (Myers *et al.* 2000; Jetz *et al.* 2012). Ecuador forms part of the tropical Andes biodiversity hotspot (Davies *et al.* 2007) and its 1628 described avian species make it one of the most bird diverse countries in the world (Ridgely & Greenfield 2006). We focused our studies on a mega diverse Amazonian bird community in Southern Ecuador which sets an ideal scenario to explore if high bird diversity promotes the evolution of generalist symbionts: in this case local *Haemoproteus* and *Plasmodium* parasites. We expanded our focus to an elevational gradient in the Podocarpus National Park which provided us a gradient in bird species diversity, with a peak diversity around 2000 meters above sea level decreasing with increasing altitude (Rahbek *et al.* 1995; Beck *et al.* 2006). Finally, we included a second Amazonian bird community, which was distinctive due to its elevated grade of bird endemism. A good system to explore our hypotheses at the interspecific scale are hummingbirds which have radiated in an astounding diversity of 338 species in the new World and have been studied extensively (Graham *et al.* 2009; McGuire *et al.* 2014; Weinstein *et al.* 2014). Still, very little is known about their parasites. Hummingbirds are a special habitat for blood parasites given their high metabolic rates, energy-demanding flight, and small body size. They possess special adaptations such as high relative heart and lung volumes, mitochondrial respiration rates, and capillary volume densities, which makes them distinctive in the vertebrate world (Suarez et al. 1991). These birds also have small erythrocytes capable of flowing through numerous narrow capillaries and possessing a high surface to volume ratio, which guarantees the quick exchange of respiratory gases (Opazo *et al.* 2005). Moreover, their blood has the highest erythrocyte level among birds, reaching values that may exceed 6.5 million red blood cells per microliter (Glomski & Pica 2011). Taken together, the explosive

radiation of hummingbirds provides an ideal system to investigate the evolution of host specificity among avian haemosporidians.

Study sites

Wisui reserve, Amazonia, Ecuador

The Wisui reserve lies in the protective forest of Cutucú-Shaimi in the East Amazonia region of Ecuador and has an extension of 311,500 hectares (Care *et al.* 2012). This protected area belongs to the Cutucú cordillera which is separated from the Andes by the rivers Zamora and Upano. Bird species richness has been estimated for over more than 480 bird species and the sampling area represents vast parches of lowland Amazonian forest (Mogollón & Guevara 2004; Santander, Freile & Loor-Vela 2009). This study was conducted around the Wisui biological station in the middle of the Cutucú mountain range in Southeast Ecuador (Macuma parroqui, Taisho canton, Morona-Santiago Province) (Fig.2). The biological station of Wisui, with an extension of four hectares and situated at a distance of approximately 500 m of the Wisui center (02°07'S, 77°44'W, 650 m altitude), has been created to develop research in the line of the international Master's program "Biodiversity in Tropical Areas and its Conservation" (www.masterenbiodiversidad.org) and consists of 3000 hectares of primary forest in excellent conservation status. The climate conditions are classified as tropical and very humid with an annual average temperature between 23 and 25,5°C and an average precipitation of 3000 mm (Cañadas Cruz 1983).

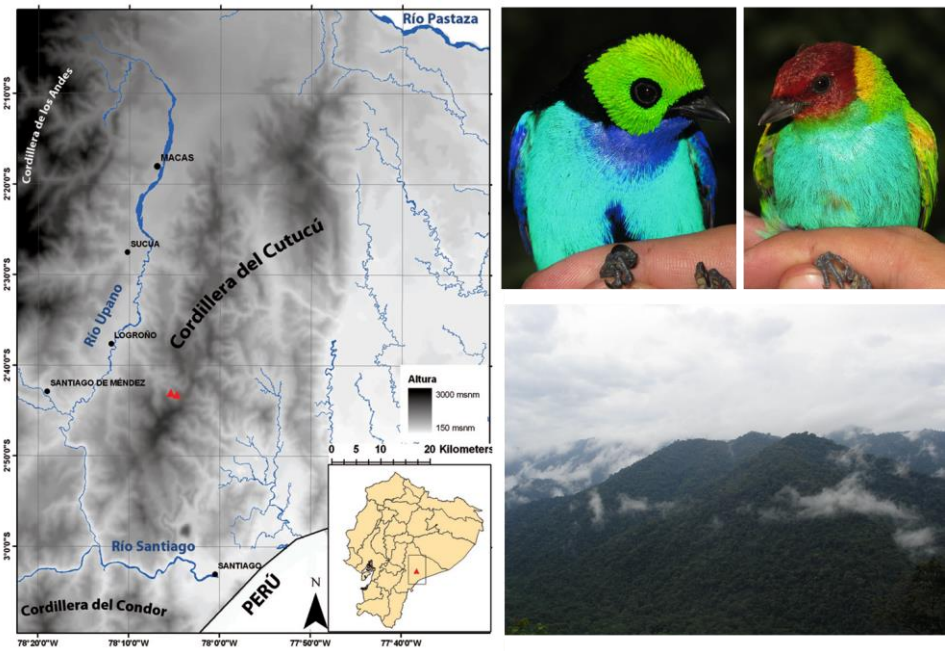


Figure 2: Map showing the field site within the Wisui reserve (left) (Brito & Pozo-Zamora 2013). Two common tanager species (*Tangara chilensis*, *Tangara gyrola*) infected with *Haemoproteus* parasites (upper right) and an overview of higher parts of the Wisui reserve (lower right).

Podocarpus National Park, Andes, Ecuador

The Podocarpus National Park extends from the Cordillera Real, the eastern Andes range, at 3800 meters towards the lowlands Amazon rainforests to the west (Beck *et al.* 2006). The Cordillera Real is a weather divide between the humid Amazon (Oriente) and the dry inter-Andean valleys. Unlike in the north of the country, where the Andes culminate at heights of 6300 meters (Chimborazo volcano), this Cordillera has a maximum height of 3800 meters and forms part of the depression of Huancabamba. The Podocarpus National Park is a biodiversity hotspot (Myers *et al.* 2000) with over 560 bird species registered (Rahbek *et al.* 1995; Santander *et al.* 2009) and is beautifully referred to as the "Andean Jewel in the Crown". We sampled birds along an elevational gradient on the western slopes of the eastern cordillera in the Podocarpus National Park at four different altitudes (Vilcambamba: 1500 meters above sea level (masl), 4°15'S, 79°13'W); Reserva "El Bosque" 2000 masl, 4°14'S, 79°10'W; Cerro Toledo: 2500 masl, 4°23' S, 79°08'W; and Cerro Toledo: 3000 masl, 4°23'S, 79°07'W) (Fig. 3). Sampling was done during five consecutive months (June-November, 2012), which started during the rainy season and ended in the dry season. The sites varied from dry tropical forest (1500 masl) to cloud forest (2000, 2500 masl), elfin forest and highland páramo (3000 masl).

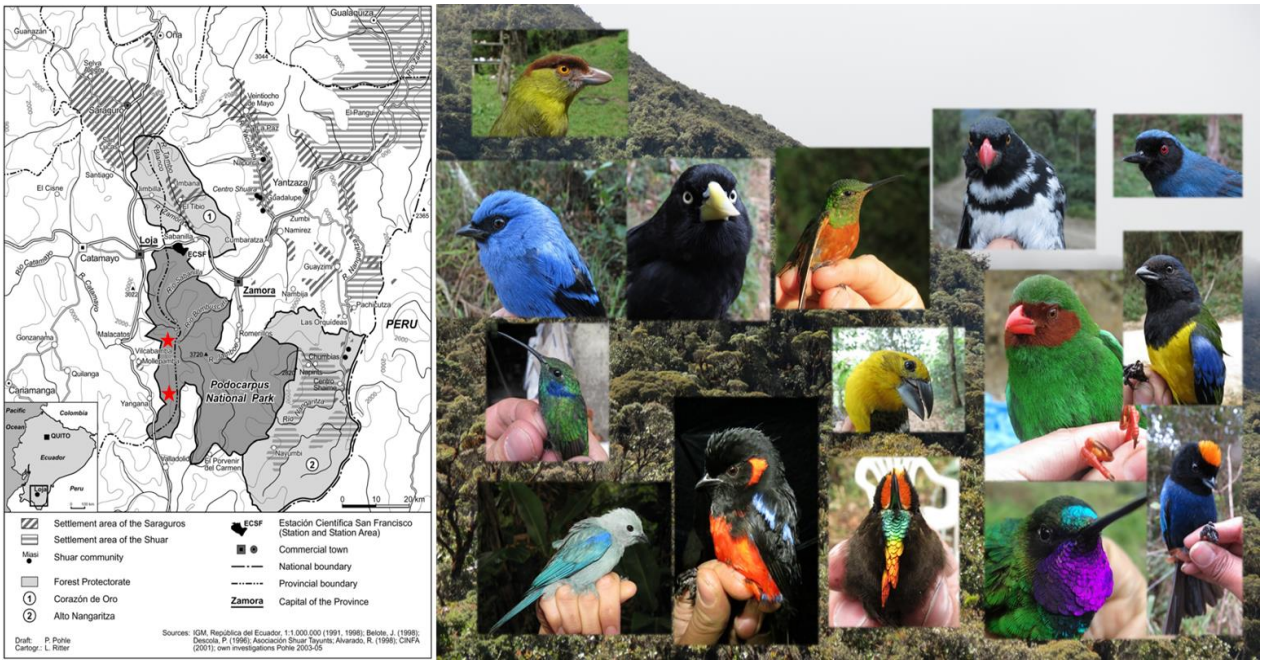


Figure 3: Location of the Podocarpus National Park (Pohle 2008) and sampling sites (red stars) (left). A sample of understory birds along an elevational gradient in the park (right).

Nouragues reserve, Amazonia, French Guiana

The Nouragues reserve (http://www.nouragues.cnrs.fr) is located in French Guiana and is part of France as an overseas department. It hosts the Nouragues ecological research station run by the CNRS (Centre National de la Recherche Scientifique) which consists of two base camps: Inselberg (4°05'N, 52°41'W) and Pararé (4°02'N, 52°41'W) (Fig.4) (Ringler *et al.* 2014). The ecosystem consists of lowland Amazonian rainforest with a complex relief of small hills and ridges (27-80 meters above sea level) which are separated by small creeks (Ringler *et al.* 2014). In the reserve, more than 480 bird species have been registered and many belong to endemic species of the Guiana Shield (Bongers *et al.* 2001).

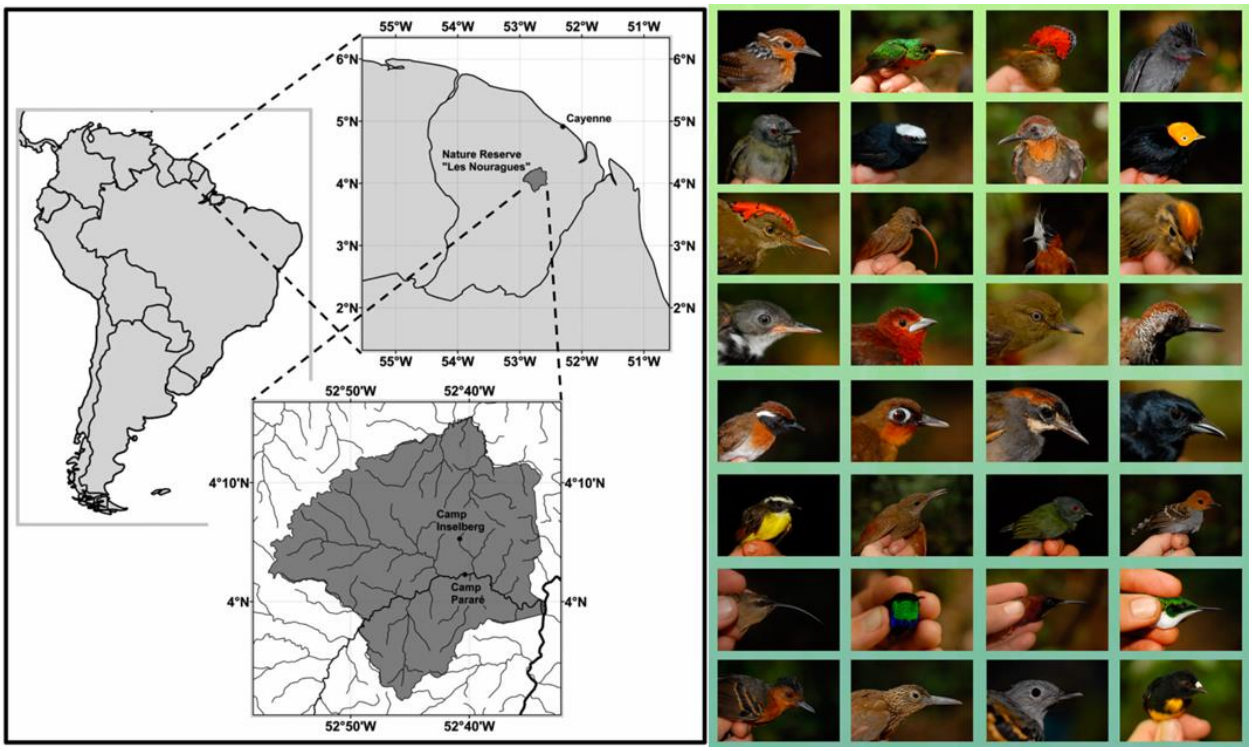


Figure 4: Location of the Nouragues reserve, French Guiana, which holds the research stations of Pararé and Inselberg (left). From (Ringler et al. 2014). A sample of understory birds of the lowland Amazonian forest (right).

Sampling methods

Bird sampling and handling

All birds were captured by the use of mist nets (12 m long, 2.5 m high, 25 mm mesh, Ecotone), which enabled the study of primarily understory birds. Birds were carefully extracted from the nets and kept in cotton bags until processing. All birds were determined with the following field guides: Aves de Ecuador (Ridgely & Greenfield 2006), Birds of Peru (Schulenberg *et al.* 2007), Birds of Colombia

(Hilty & Brown 1986), Birds of Northern South America (Restall, Rodner & Lentino 2007) and updated according to checklist of the South American Classification Committee (Remsen *et al.* 2016). We banded the birds with a unique numbered plastic or aluminum rings. Hummingbirds were marked with a feather clip code by cutting a tip of the 1st-6th primary wing feather in order to control for recaptures. The following data were taken from each individual: species, age, sex, tarsus length, beak length, wing length, tail length, fat and muscle content, presence of brood patch and cloacal protuberance, mold status and weight. All birds were photographed in order to confirm species identification a posteriori. After handling the birds were released unharmed at the site of capture. A total of 1295 birds and 307 birds were captured in Ecuador and French Guiana respectively. We captured and manipulated birds under a bird ringer license (nr. 530354) provided by the Spanish Ministry of Environment. All birds were handled for short time periods (less than 10 minutes) in order to minimize stress. Captured individuals were released immediately after sampling. We did not collect any bird specimens. The protocols described in this document were carried out following the guidelines by the Neotropical Ornithological Society's "Guidelines to the Use of Wild Birds in Research." (http://www.nmnh.si.edu/BIRDNET/guide/). All sampling procedures and manipulation experiments used in this study were reviewed and approved by the Ministry of Environment of the Loja Province in Ecuador (N°009-2012-IC-FAU-DPL-MA) which acts as a local Animal Ethics Committee. The sample collection permits were provided by the Ministry of Environment of the Loja province under the research project N°009-2012-IC-FAU-DPL-MA. The samples were transported under the following export permits: CITES 021/VS and 016-2012-IC-FLO-DPL-MAD provided by the Ministry of Environment of Ecuador and Spain respectively. In French Guiana, permits provided by the Centre National de la Recherche Scientifique (CNRS) and by the 'Direction Régionale de l'Environment de Guyane' (DIREN) covered all bird handling procedures and animal ethics guidelines under the WOODNYMPH project.

Blood parasite sampling and laboratory analyses

After measuring the birds, blood was extracted from the brachial or jugular vein in the case of passerines and bigger birds. Hummingbirds were sampled by a puncture in the metatarsial vein (Fig. 5). The puncture was performed with insulin needles (0.3 mm x 12mm; Omnican U-100, BRAUN, Bad Arolsen, Germany) and 20-100 µl was collected in vials with absolute ethanol until further analyses. Two blood slides were prepared for microscopy analyses and were air dried and fixed in absolute ethanol within 24 hours. Blood smears were stained with GIEMSA solution (pH 7.2) during 60 min. Each blood smear was observed with a light microscope (LEICA DM2500). We first scanned smears at relatively low magnification (400×) to cover the whole smear in the search for parasites. Then we screened at 1000× focusing on intra-erythrocytic parasites, until ca. 100.000 erythrocytes were inspected.

Total DNA from blood samples was extracted with a standard ammonium acetate protocol (Green et al. 2012). We amplified the MalAvi barcode for avian haemosporidians (479 bp of the cytochrome b gene; (Bensch et al. 2009)), which has been sequenced for the majority of the known diversity of these parasites and facilitates the analysis of their evolutionary relationships. This sequence identifies unique parasite lineages and thus serves as a barcode for species recognition (Bensch et al. 2009). The MalAvi database provides access to lineage information on haemosporidian infections collected from over 200 publications and can be viewed on the MalAvi website (http://mbio-serv2.mbioekol.lu.se/Malavi/;(Bensch et al. 2009)). All samples were checked on quality for PCR by amplifying bird sexing markers (Fridolfsson & Ellegren 1999). We screened for parasite infections using the nested PCR protocol developed by Waldenström et al. (2004), which was specifically designed to amplify *Haemoproteus* and *Plasmodium* DNA. In the second chapter we used a protocol which amplifies *Haemoproteus*, *Plasmodium* and *Leucocytozoon*, which amplifies the same MalAvi barcode for haemosporidians (Hellgren, Waldenström & Bensch 2004). All sequences were aligned with the BioEdit software (Hall 1999) and carefully revised until further analyses. Chromatograms were analyzed in detail for signs of double infections (Pérez-Tris & Bensch 2005). New lineages were added to the MalAvi database and uploaded to Genbank.

Figure 5: Extraction of a blood sample from (A) the metatarsial vein from the great-billed hermit (*Phaethornis malaris*), (B) the brachial vein of a paradise tanager (*Tangara chilensis*), (C) the jugular vein of the buff-throated saltator (*Saltator maximus*) and (D) the brachial vein of the white-plumed Antbird (*Pithys albifrons*).

Virus sampling and laboratory analyses

All birds were carefully screened for the presence of nodular cutaneous lesions on the feet, legs and beak. When present, we collected part of the lesion by excision of a biopsy to maximize detectability of viruses by PCR from tissue DNA (Williams *et al.* 2014), and stored it in sterile tubes with absolute ethanol. When symptomatic birds were handled, sterile gloves and scalpels were changed to avoid possible cross-contamination. The individual lesion samples were weighted, homogenized by mechanical force using a sterile plastic crusher, and total DNA was extracted with a standard phenol-chloroform-isoamyl alcohol protocol followed by isopropanol precipitation (Pérez-Tris *et al.* 2011). DNA extracts were controlled for sample quality on a 1% agarose gel, and DNA concentration was measured with a Nanodrop ND100 system (Nanodrop Technologies, ThermoScientific, Wilmington, DE) and stored at -20 °C. Samples were first tested for possible PCR inhibition by amplifying a fragment of the bird cytochrome *b* gene (Pérez-Tris *et al.* 2011) as a control, and all six tissue samples produced positive amplification. Then a multiplex PCR designed for the combined detection of *Avipoxvirus* and *Papillomavirus* was performed under conditions described by Pérez-Tris et al. (2011). Samples which tested positive for *Avipoxvirus* were visualized on a 2% agarose gel by the appearance of a DNA fragment of approximately 250 bp, and were further analysed by amplifying 448 bp of the P4b core protein gene (Pérez-Tris *et al.* 2011). This PCR was performed under the same conditions as the multiplex PCR and products were visualized on a 2% agarose gel stained with ethidium bromide. Positive samples were sequenced by Macrogen (Netherlands) and were compared with known P4b sequences available from GenBank. Both CRESS DNA genomes were amplified by performed a rolling circle amplification (RCA) in order to amplify circular DNA genomes from the *Avipoxvirus* lesion (Dean *et al.* 2001; Johne *et al.* 2009). After the RCA reaction the product was digested with the EcoRI enzyme. Four DNA fragments with different intensities were extracted from the agarose gel (QIAquick Gel Extraction Kit, QIAGEN, Germany) and cloned and sequenced. A wide variety of walking primers and back-to-back primers were designed for the amplification and sequencing of both virus genomes.

Results and Discussion

Evolution of host specificity

In **chapter 1** we set out to explore if in a megadiverse bird community in the Southeastern Ecuadorian Amazon a generalist strategy of host exploitation is promoted in local *Haemoproteus* and *Plasmodium* assemblages. We then took a closer look on how generalist parasite lineages may have evolved in this area by studying their phylogenetic distribution within the known diversity of blood parasites. Finally, we compared the host specificity of the local parasite assemblages in Ecuador with multiple parasite communities across the world, in order to test if they are significantly more generalist than elsewhere, with special focus on temperate areas. We analyzed local and global host range by the use of a well-known host specificity index S^*_{TD} (Hellgren et al. 2009).

We compared the mean host specificity index S^*_{TD} of the local blood parasite communities in Ecuador with 20 and 27 parasite communities, for *Plasmodium* and *Haemoproteus* respectively, throughout tropical and temperate areas. The mean host specificity of *Haemoproteus* was significantly more generalist than 15 out of 21 communities sampled outside of South America (Fig. 6). Within South America, no significant difference in host specificity was found with seven other *Haemoproteus* communities (Fig. 6). In contrast, no apparent geographical pattern was found in the variation of host specificity of *Plasmodium* parasite communities (Fig. 6). We thus found very strong support that South American *Haemoproteus* communities are more generalist than elsewhere. It is however surprising that *Haemoproteus* communities in other tropical areas in Africa and Asia are not more generalist. According to the dilution effect hypothesis, natural selection should favor the evolution of generalists when facing high host diversity. This difference in host specificity between tropical areas could be due to several reasons. The first one is methodological: many places were studied on a broader geographical scale compared to our sample, with a small sample size on many locations. This could somewhat decrease the calculated host specialization. Vector-feeding preferences could also shape the evolution of parasite host specificity (Gager *et al.* 2008; Njabo *et al.* 2011). If vectors have more specialist feeding patterns, the chances of host-switching would decrease giving and evolutionary advantage to specialist parasites. On the other hand, if *Haemoproteus* vectors would be more generalist, this would increase host encounter rate, giving an evolutionary advantage to generalist parasites. Future research on the vector diversity and feeding patterns is thus critically needed to understand how host specificity evolves in this parasite system.

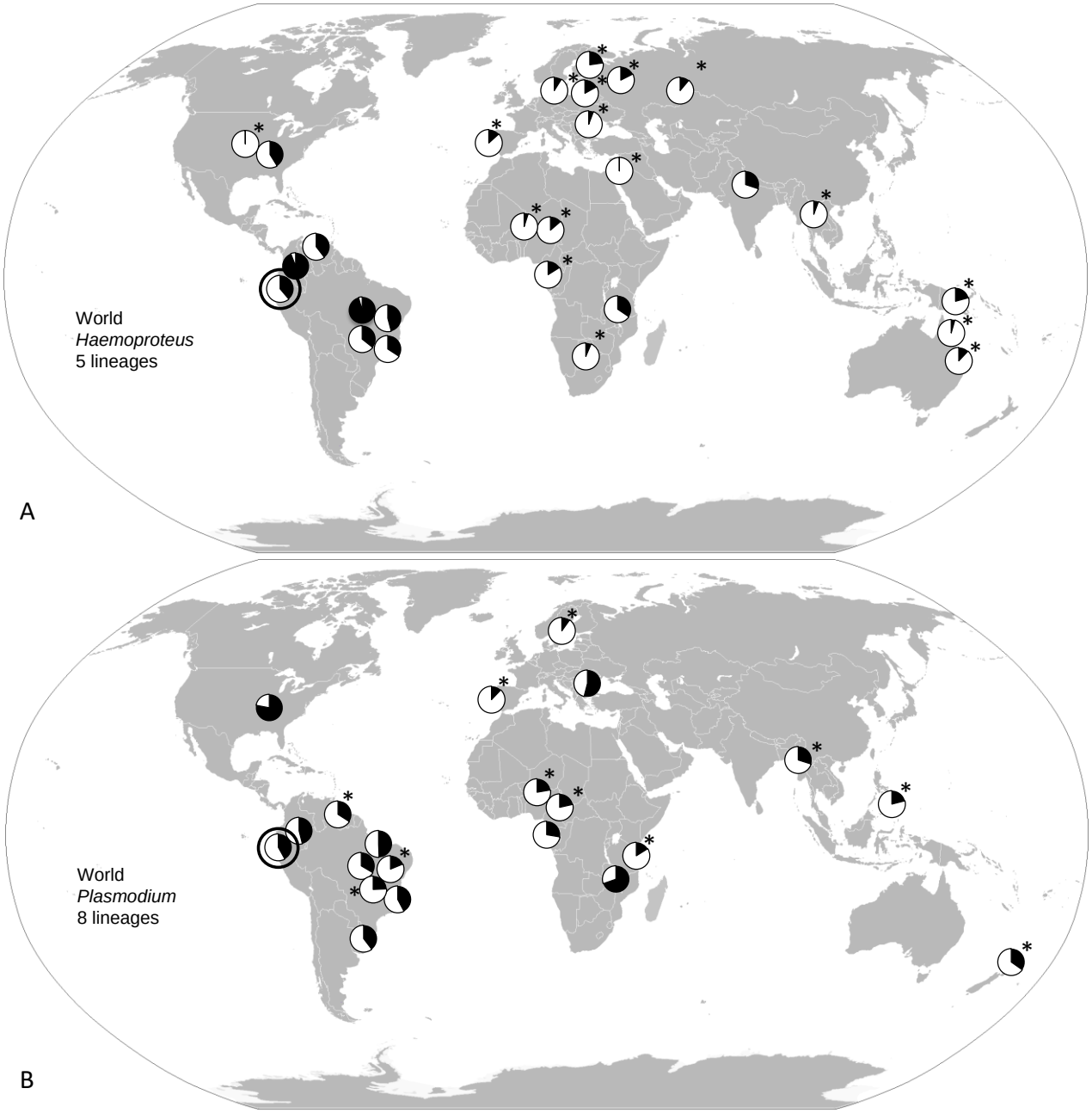


Figure 6: Variation in the mean host range of randomly assembled communities of (A) *Haemoproteus* (5 lineages) and (B) *Plasmodium* (8 lineages) in South America and the world, as obtained from sampling sites where local S^*_{TD} indexes (mean host specificity value) could be computed based on published data. This shows that *Haemoproteus* communities are more generalist than other communities outside the Neotropics, while no clear pattern is apparent for *Plasmodium*. In all graphs, the host range of the parasite community is shown in black, assuming a full circle represents $S^*_{TD} = 9$. Statistically significant differences between our sample in Ecuador (encircled) and other sites are marked with stars.

In our bird community in Ecuador, both *Haemoproteus* and *Plasmodium* show a wide phylogenetic ancestry within the known parasite diversity, but this was greater for *Haemoproteus* than for *Plasmodium*, which diversity was concentrated in two major clades (Fig. 7). Half of the *Haemoproteus* lineages were generalist and were the most closely related with specialist parasites or have evolved from a specialist ancestor (Fig. 7). Generalist *Plasmodium* lineages evolved from a generalist ancestor but were most closely related to specialist parasites (Fig. 7). These results show

that the local *Haemoproteus* diversity is the consequence of multiple colonization events rather than from local parasite radiations and that host switching has played a major role in the assemblage of this unique blood parasite community (Fig. 7). The fact that all generalist *Haemoproteus* lineages evolved from a specialist ancestor suggests that a generalist strategy evolved multiple times in this megadiverse community. The driving force of the evolution of generalist parasites remains an open question but points towards the importance of relative host diversity and abundance. In tropical ecosystems where host diversity is the highest, the prevalence of a parasite that infects one single host species can be reduced by dilution effects, yet the high diversity of hosts reduces the chances for the parasites to be transmitted into the appropriate host (Keesing *et al.* 2006). When parasites manage to live successfully in a wide array of host species, their prevalence may increase as a result of the reduction of this dilution effect and the increase of the host encounter rate.

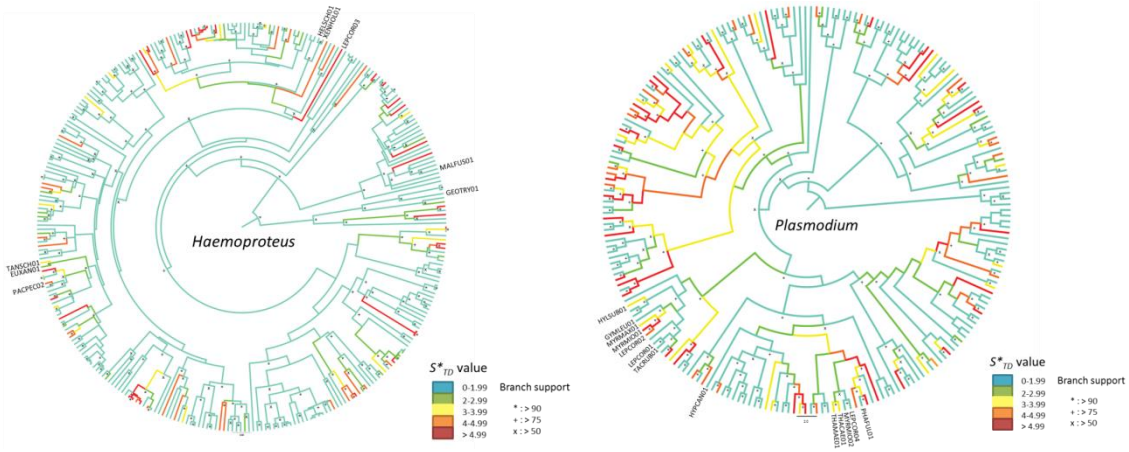


Figure 7. Both *Haemoproteus* and *Plasmodium* lineages in Ecuador (marked with lineage names), show a wide phylogenetic ancestry within the worldwide parasite diversity. This shows the local parasite assemblages are the result of multiple colonization and host switching events rather than local parasite radiations, which is more pronounced for *Haemoproteus*. Generalist *Haemoproteus* parasites (distinct than blue) are closely related to specialists (blue branches) or descended from a specialist ancestor. This suggests that *Haemoproteus* parasites evolved a generalist strategy multiple times upon the colonization of the megadiverse bird community in Ecuador.

Host specialization in unique habitats

In **chapter 2** we explored if the unique hummingbird niche is exploited by generalist or specialist *Haemoproteus* parasites. This was approached by asking if (a) the *Haemoproteus* community of hummingbirds is the result of multiple colonization events, (b) if the found *Haemoproteus* parasites are shared with other birds orders and (c) to what extent hummingbirds are exploited by these parasites in terms of prevalence and infection intensity.

All *Haemoproteus* parasites in hummingbird were generalists, shared among different hummingbird species and passerines. The *Haemoproteus* lineages showed a wide phylogenetic ancestry within the diversity of *Parahaemoproteus* indicating that the assemblage of this unique community of parasites

is the result of multiple colonization events and that host-switching has been important in shaping the hummingbirds parasite diversity (Fig. 8)

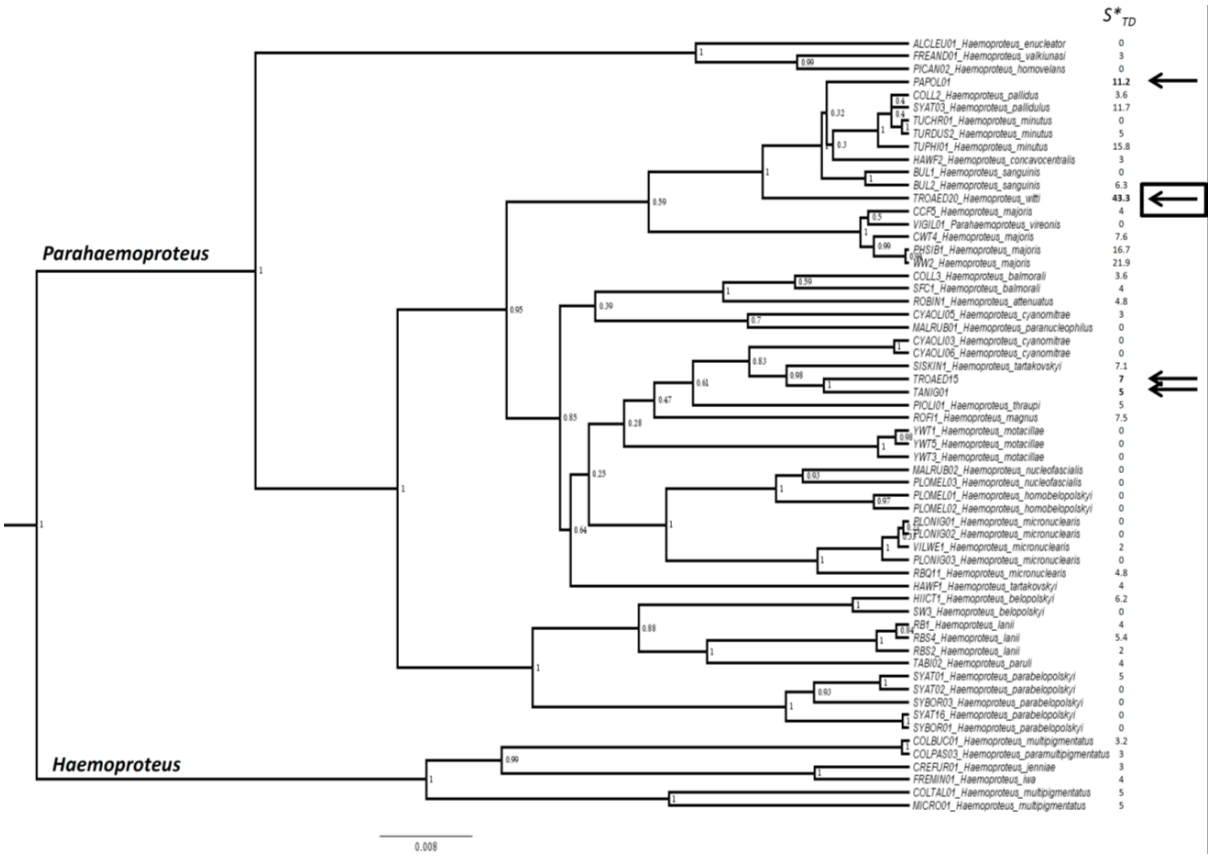


Figure 8. Evolutionary relationships of hummingbird *Haemoproteus* lineages with morphologically and molecularly identified *Haemoproteus* species worldwide. The four *Haemoproteus* lineages (marked with arrows) are widely distributed among the *Parahaemoproteus* phylogeny indicating that host-switching played a major role in the evolution of the hummingbird parasite community. *Haemoproteus witti* (marked with a black box) is the most generalist *Haemoproteus* parasite found to date, according to PCR diagnostics and the S^*_{TD} host specificity index.

To explore to the importance of hummingbirds as reservoirs for *Haemoproteus witti* we measured prevalence and infection intensity with microscopy and PCR techniques for the most prevalent *Haemoproteus* parasite in this community. We found a positive relationship between infection intensity measured by light microscopy and infection intensity measured by qPCR (Fig. 9 A and B). This indicates that both techniques are consistent. Moreover, a positive relationship was found between the mean infection intensity measured by qPCR and the prevalence of *Haemoproteus witti* across all infected host species (Fig. 9 C). As observed, hummingbirds show the highest infection intensities and prevalence across all hummingbird species and passerine species, which suggest that hummingbirds are the most relevant reservoirs for *H. witti* both in terms of prevalence and gametocymia.

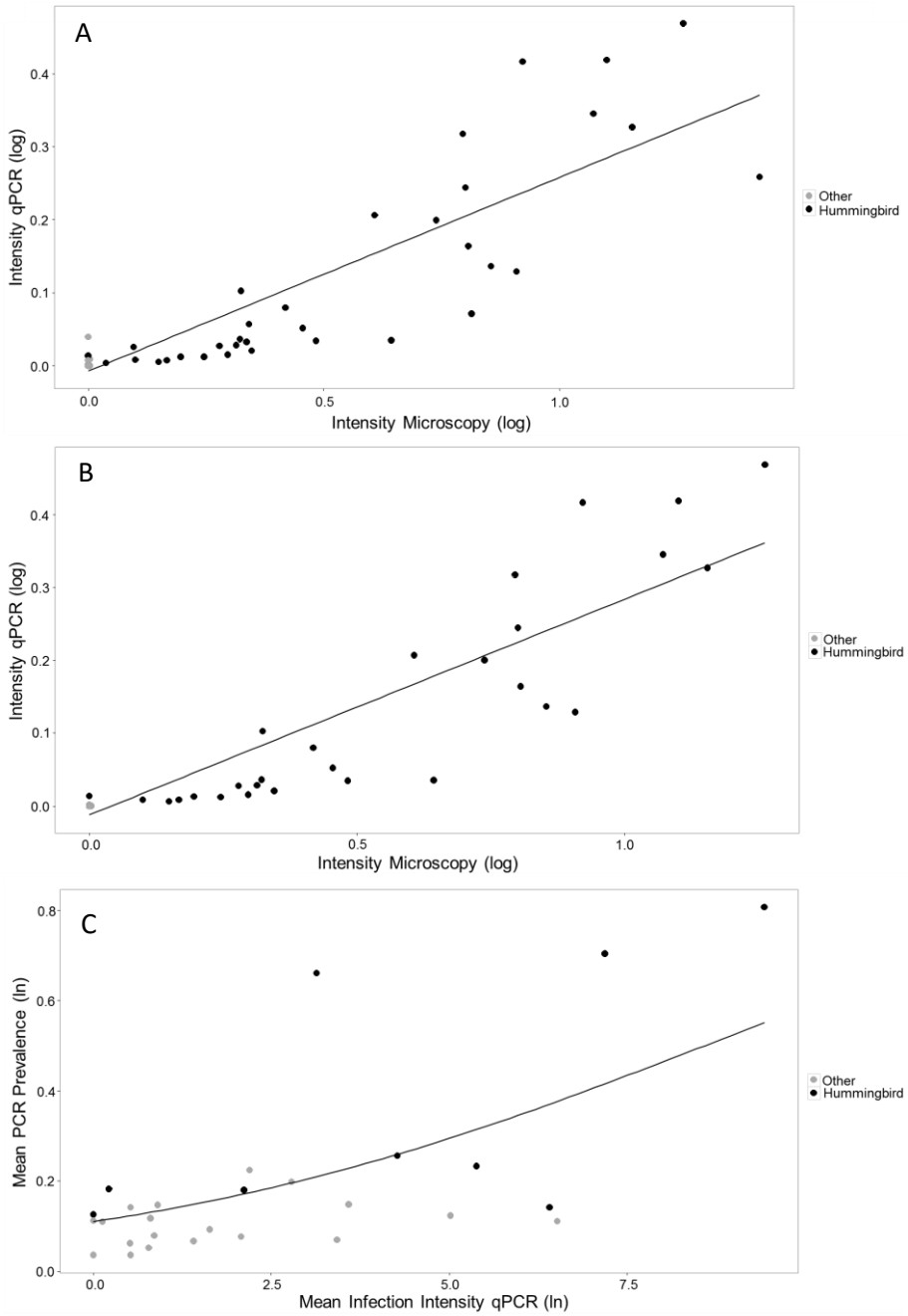


Figure 9. Graphs A and B show the relationship between parasitaemia of *Haemoproteus witti* (the most generalist and prevalent parasite) measured by microscopy and qPCR. In (A) all infected individuals are included in the analysis while in (B) all individuals co-infected with *Leucocytozoon* were excluded. These relationship shows that both microscopy and molecular detection techniques give equal results and are good techniques to measure parasite infection intensity. Moreover, it shows hummingbirds have higher parasitaemia than other birds (passerines). Graphs C shows the correlation between mean infection intensity (as determined by qPCR, bootstrap means) and prevalence (as derived from PCR data, bootstrap means). This uncovers hummingbirds as the most relevant reservoir for the transmission of *H. witti*, both in terms of prevalence and infection intensities. Hummingbirds are represented by black dots while other species are depicted as grey circles. All correlations are highly significant ($P \leq 0.01$).

These results uncover the evolution of a unique community of avian blood parasites, composed of generalist parasites with different dependence on hummingbirds as their main hosts. Our results challenge the current view we have on host specialization in blood parasites. In most studies

nowadays, host specificity is measured based on the presence of parasite DNA in the bird blood, measured by PCR techniques. Here we show that *H. witti* exhibits very different infection intensities in different hosts. In passerines the gametocymia is so low compared to the one in hummingbirds that one could question their importance as reservoirs for this parasite. From this perspective, this generalist parasite can be viewed as a hummingbird specialist. Future research will show if *H. witti* can successfully complete its life cycle in passerines. Meanwhile we intent to present a hypothetical model for the evolution of *H. witti*'s host specificity (Fig. 10).

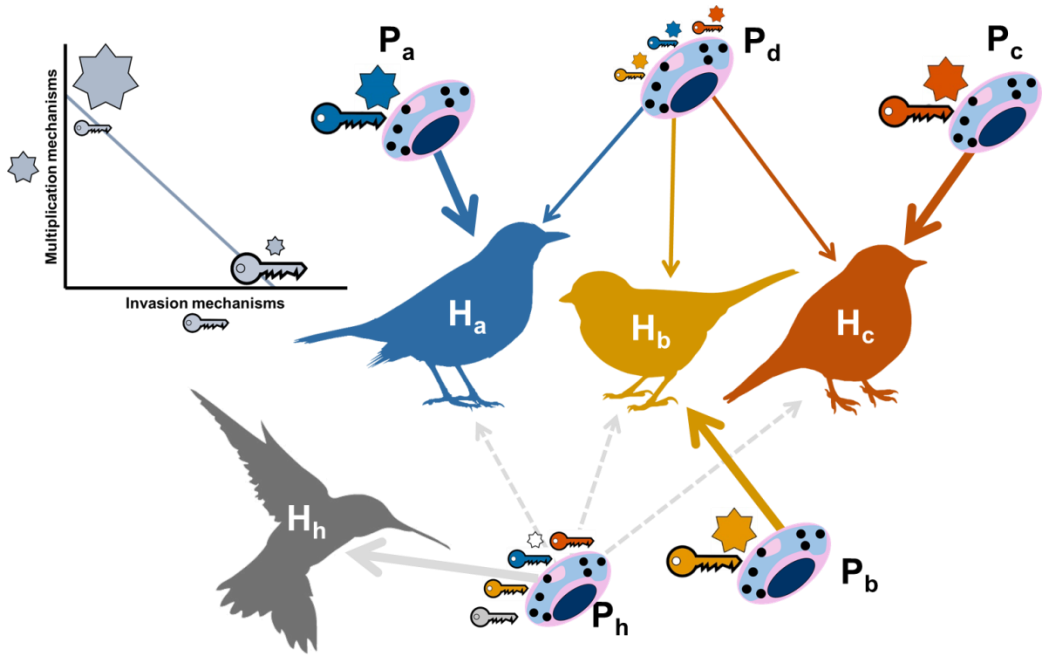


Figure 10. Parasite exploitation mechanisms are assumed to be multiple, costly, and constrained by trade-offs. In the model, any increase in investment in mechanisms that facilitate host invasion (keys) is associated with reduced investment in mechanisms promoting parasite multiplication in the host (such as immune evasion, stars). Birds have evolved costly immune mechanisms (represented by colour shades). Only parasites that evolved mechanisms to successfully invade and evade the immune system of the different host species can establish and successfully multiply in these hosts. Parasites Pa, Pb and Pc have become specialists by investing much in mechanisms that allow successful exploitation of host species Ha, Hb and Hc, respectively. High success of these parasites is represented by thick arrows linking them with their hosts. One parasite that distributed a similar amount of resources among multiple invasion and evasion mechanisms to exploit various species has become a generalist (Pd), which is less successful (narrow arrows) in its hosts than the corresponding specialist. Another parasite (Ph) has evolved an alternative strategy, which promotes host invasion at the expense of immune evasion mechanisms. Not investing in costly evasion mechanisms (empty star) constraints its ability to multiply in the majority of host species (broken arrows), even if it still can invade them. In the tropics, where high host diversity may promote multiple invasion or evasion mechanisms among local parasites, this may be a way of specializing in hummingbirds (Hh), whose energetically costly lifestyle may compromise their immune capacity, thereby giving a chance to parasites with high host invasion potential.

The observation of extreme high parasitaemia in hummingbirds was unexpected and opens new lines of research. How can birds which depend on high metabolism cope with these extreme infection intensities? Are tolerance or resistance mechanisms key in understanding how they survive these infections? A possible explanation is that hummingbirds rely more on tolerance mechanisms rather than resistance to maintain fitness under heavy parasite pressure, while other birds could easily resist

infection by *H. witti* by mounting costly immune defenses (Bonneaud *et al.* 2003). Tolerance could be measured by plotting infection intensity against the host fitness of multiple host individuals of different species and by exploring the slope of this regression; the steeper the slope, the lower the tolerance of a host species to a given parasite (Råberg, Sim & Read 2007; Råberg, Graham & Read 2009). If hummingbirds present tolerance mechanisms against *H. witti*, this parasite could be locally successful without having to invest in costly immune evasion mechanisms. Non hummingbirds could present resistance mechanisms against *H. witti* without compromising the transmission of the parasite. If this proves true we could show the existence of a new mechanism of parasite specialization in a special niche.

The nature of emerging diseases

In **chapter 3** we specifically looked for key characters of *Avipoxvirus* which make them good candidates to cause a disease outbreak under favorable ecological and evolutionary circumstances. Furthermore, we explored the diversity and host specificity of *Avipoxvirus* lesions in two wild bird populations in the Neotropics.

In Ecuador we found three infections of two newly *Avipoxvirus* strains in three different bird species (Fig. 11). Both new strains belong to the canarypox clade (Fig. 11). The new strain THREPI01 is most closely related to a strain found in the Madeira archipelago while strain THRCYA01 belongs to an America clade of avipoxviruses (Fig. 11). These results emphasize that *Avipoxvirus* in the Neotropics circulate at low levels in wild bird populations and that the diversity has multiple geographical origins. The host specificity analyses indicate that host-switching was important in shaping the evolution of host-parasite interactions in this system. Most *Avipoxvirus* strains are characterized by their generalist nature and by the fact they are widely distributed over continents. *Avipoxvirus* strains are transmitted on a worldwide scale, particularly strains belonging to the fowlpox clade (Fig. 11). Moreover, sister strains in the canary- and fowlpox clade are mostly found in distant continents, indicating that extensive host and distribution shifts have taken place in the evolutionary history of these viruses. In summary, our study supports the idea that *Avipoxvirus* circulates at a low prevalence and diversity at the community level in forest understory of lowland Amazonia in French Guiana and the cloudforests in South Ecuador. Nevertheless, most *Avipoxvirus* strains show high dispersal capacity and low host specificity and could spread to new host species when particular circumstances occur. The combination of naïve hosts with a short coevolutionary history with invasive parasites, adequate climatic conditions to maintain infective vector populations, and a reduced diversity of hosts, provide favorable conditions for the emergence of *Avipoxvirus* in new environments. Additional sampling of birds in the Neotropics and elsewhere is needed to understand the variation in host specificity and distribution of *Avipoxvirus* strains in order to recognize the value of biodiversity

in suppressing disease emergence and its effects on the transmission of *Avipoxvirus* in wild populations of birds.

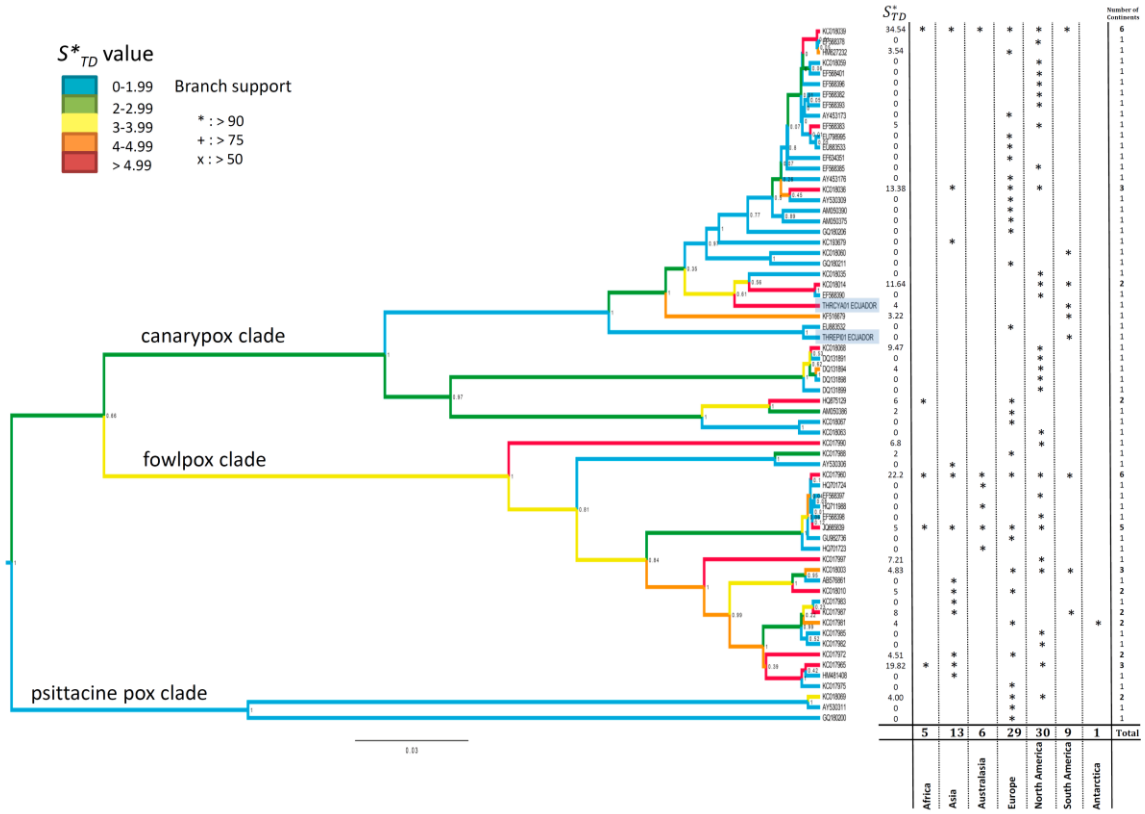


Figure 11: Evolutionary relationships of the two new *Avipoxvirus* strains found in this study (highlighted in grey) with the worldwide *Avipoxvirus* diversity (as of 2015). Both strains present distinct evolutionary trajectories within the canarypox clade, indicating multiple colonization events of the local avifauna in Ecuador. Closely related strains are found on various continents, showing these viruses have high capacity to circulate globally, especially strains belonging to the fowlpox clade. The host specificity index (S^*_{TD}) indicates that many strains are generalist (coloured branches distinct than blue), which seems to be more pronounced in the fowlpox clade. These analyses emphasize the generalist nature of *Avipoxvirus* and their facility to move across wide geographical areas. Strains found in more than one continent are highlighted in bold.

Biodiscovery

In **chapter 4** we characterize two novel CRESS DNA viruses we detected on the *Avipoxvirus* lesion of the blue-and-gray tanager (*Thraupis episcopus*) in South East Ecuador. After performing rolling cycle amplification on the lesion in order to find possible infections of *Papillomavirus* we amplified two unknown circular viruses. We found that these viruses belong to the megadiverse group of CRESS DNA viruses. Two new CRESS DNA viruses were amplified from the *Avipoxvirus* lesion. We refer to them as Tanager-associated CRESS DNA virus TaCV1 (3401 nt bases) and TaCV2 (2967 nt bases). TaCV1 shows an ambisense genome organization with a replication-associated protein (Rep) of 393 amino acids in sense direction and one open reading frame (ORF1) of 208 amino acids in the antisense direction (Fig. 12). TaCV2 presents an ambisense genome organization with two

ORFs (ORF1, ORF2) in ambisense direction of 166 and 174 amino acids respectively and a replication-associated protein (Rep) of 321 amino acids in sense direction (Fig. 13). Both genomes also have a hairpin loop structure (5´-CAGTATAC-3´ for TaCV1; 5´-TAATACTAT-3´ for TaCV2) which represents the origin of rolling circle amplification in single-stranded DNA viruses (Fig. 12).

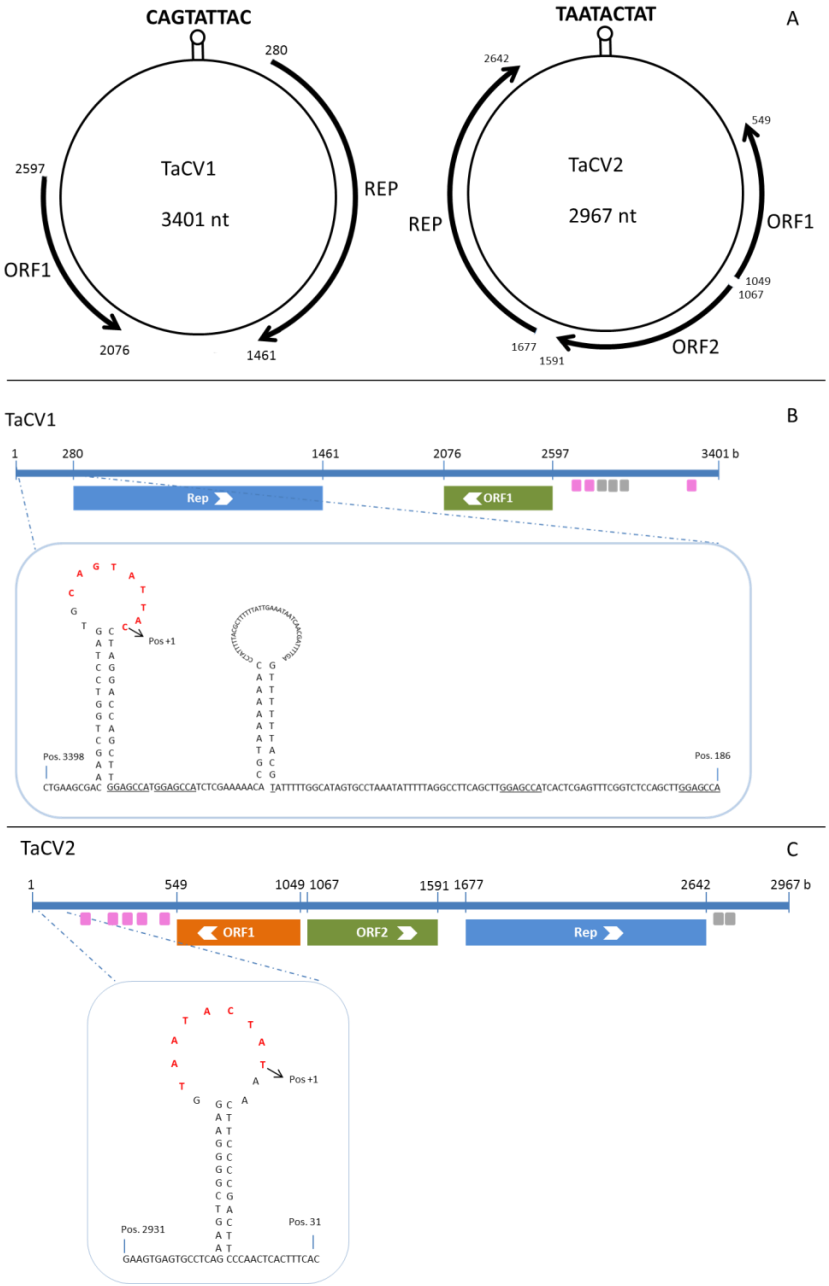


Figure 12: Ambisense genome organization of the two new CRESS DNA viruses presented in this thesis. Tanager-associated CRESS DNA virus 1 (TaCV1) represents two main open reading frames (ORF): one associated with a Replication protein (REP) and ORF1 is putatively associated with a Capsid protein. Tanager-associated CRESS DNA virus 2 (TaCV2) shows three ORFs; one associated with a Rep function and ORF1 and ORF 2 with unknown function although the latter is possibly associated with a Capsid function.

We also explored the structural function of novel putative capside proteins in order to understand the structure of the two novel gemomes. We specifically looked for the presence of intrinsically

disordered regions (IDRs), which are regions within a protein that lack an ordered structure (He *et al.* 2009). These IDRs allow a protein to exist in different states depending on the substrate they interact with (Dunker *et al.* 2001) and possibly reveal the existence of Cap proteins in CRESS DNA viruses (Rosario *et al.* 2015). Within these IDRs we also detected the presence of protein binding regions which are disordered in isolation but which can undergo disorder-to-order transition upon binding (Mészáros, István & Dosztányi 2009). We found a new characteristic IDR profile for both viruses for ORF1 in TaCV1 and ORF2 in TaCV2 with a peak in predicted disorder from residue 50 onwards, which were different to the profiles presented in Rosario et al. 2015. ORF1 of TaCV2 shows a peak approximately between residues 110 and 140, which equally represents a new IDR profile for CRESS DNA viruses. Along with the characheristic IDR profiles, motifs associated with rolling circle replication, nuclear localization signals (NLS) were found providing additional evidence for the existence of putative Cap protein in these CRESS DNA viruses. This approach revealed knowledge to understand the diversity of mechanisms and biological traits of this widespread and megadiverse group of viruses.

A Bayesian analysis placed the Rep protein of TaCV1 as a sister clade to a wide diversity of CRESS DNA viruses, in which the Rep protein of TaCV2 is embedded (Fig. 13). The Rep protein of TaCV2 is most related to Rep proteins of bat circoviruses (Fig. 13). Since closely related viruses have been isolated from very distantly related host species (Fig. 13), we show evidence that extensive host switching between phylogenetically distant hosts has governed the evolutionary history of CRESS DNA viruses, which make these viruses good models to study the evolution of host specificity. This is especially important to understand their potential of colonizing new habitats and to monitor possible disease outbreaks.

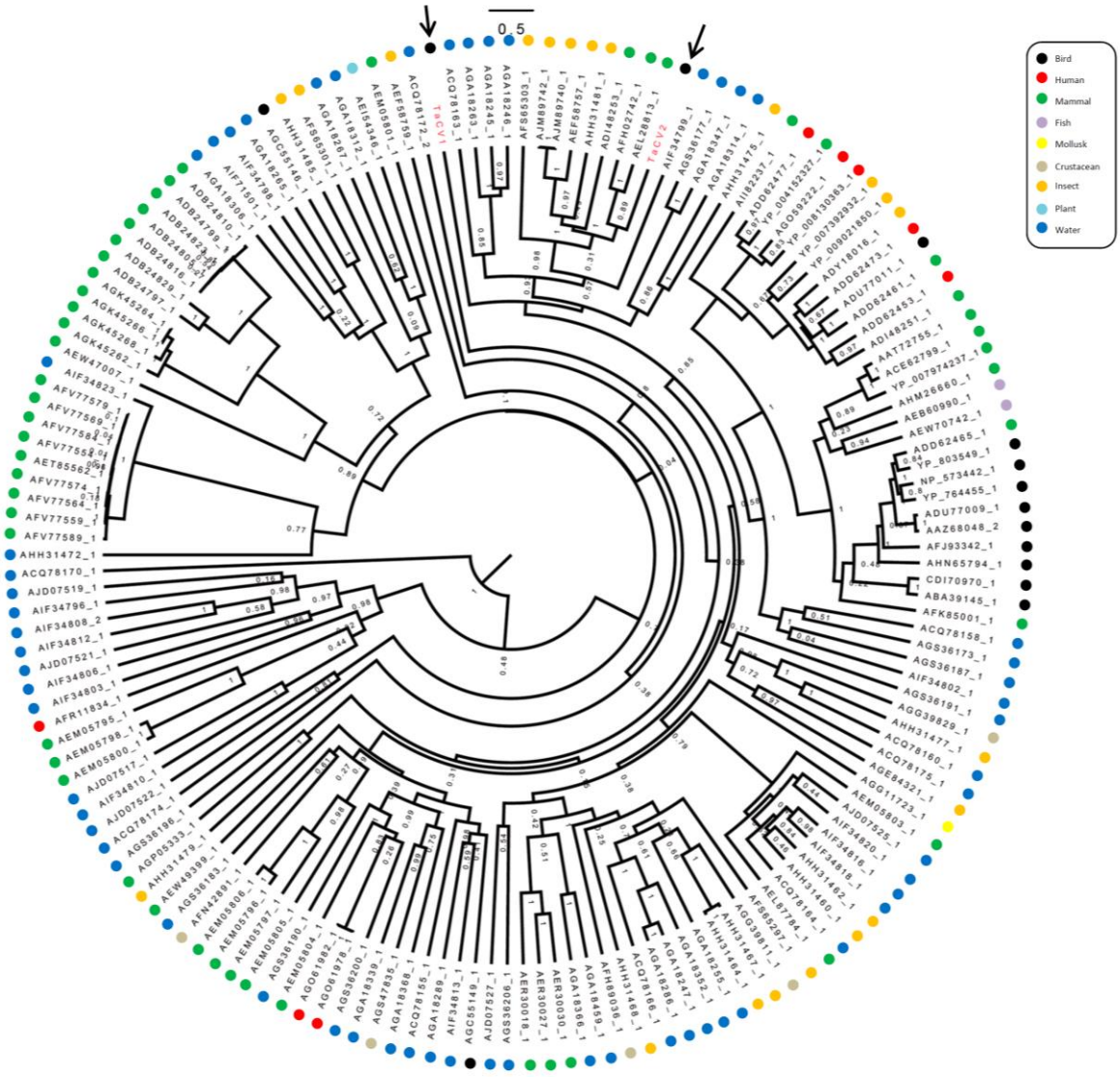


Figure 13: Evolutionary relationships of Rep proteins of the two genomes characterized in this thesis (TaCV1 and TaCV2; marked with arrows) inferred with Bayesian analyses. The colors at the end of the branches represent the host group or habitat from which the virus was isolated, showing extensive host switches have governed the evolution of CRESS DNA viruses. Numbers along the branches represent branch support (posterior probabilities).

General discussion

In this thesis, we aimed to identify historical and ecological determinants acting on the evolution of host specificity in avian blood parasites and viruses. On the one hand we pursuid to explore the importance of host-switching and strict cospeciation in the assemblage of symbionts at the ecosystem scale, by analysing different bird communities in the Neotropics. This was later refined to an interspecific scale by studying the evolution of specialization (niche filling) of blood parasites in hummingbirds, whose spectacular diversity provided an unique evolutionary scenario. On the other hand we investigated the possible importance of host diversity as an ecological selection pressure on the evolution of host specificity in avian blood parasites, by studying different bird communities across the world who varied in their diversity. At the interspecific scale, hummingbirds offered unique host characteristics in their physiology which played a role in determining the evolution of their distinct parasite communities, mainly in their importance as reservoirs for generalist blood parasites. We showed that host switching has played a major role in shaping the host-symbiont relationships between birds, avian blood parasites, avipoxviruses and CRESS DNA viruses. Finally this thesis provided an excellent opportunity to increase our knowledge about the diversity of symbiotic lifestyles in the Neotropics, which generated new models for future studies on the evolutionary ecology of symbiont specificity in megadiverse environments.

Ecosystem diversity and host specificity

The Neotropics presented an ideal playground to explore the relationship between host specificity and host diversity, as the latter could govern the evolution of host specificity through the costs and benefits of dilution and amplification effects. We found explicit evidence that *Haemoproteus* communities are more generalist in the Neotropics than in temperate areas. Phylogenetic analyses demonstrate multiple generalist *Haemoproteus* lineages evolved when facing a megadiverse bird community in Ecuador. This results reveal the Neotropics as a hotspot for generalist *Haemoproteus* parasites, which could cause serious problems if they would colonize new environments (Ewen *et al.* 2012). It is not the first time South America shows distinct patterns in avian blood parasite composition compared to the rest of the world. A recent study showed that in general *Haemoproteus* diversity exceeds *Plasmodium* diversity in all continents, except for South America, where the opposite is true (Clark, Clegg & Lima 2014). The different biogeographical patterns of *Plasmodium* and *Haemoproteus* suggest fundamental differences in how lineages diverge in both genera. Lineage diversity may result from the degree of host specialization, a strategy that promotes resource partitioning and facilitates species coexistence (Novotny *et al.* 2002, 2006). Previous research has shown that *Haemoproteus* parasites seem to be more specialized than *Plasmodium* (Ishtiaq *et al.* 2007; Beadell *et al.* 2009), which seems to explain why *Haemoproteus* diversity is generally higher (Clark *et al.* 2014). We found that *Haemoproteus* communities in South America are more generalist,

even more than *Plasmodium*, which could explain why *Haemoproteus* is less diverse than *Plasmodium* in this region. The relationship between host specialization and species diversification is a promising avenue to explore in future work.

Is the evolution of a high amount of generalist parasites a consequence of the cost of dilution effects for specialist parasites? We did not find a causal relationship between host diversity and host specificity of local blood parasite communities, so the conclusion if high host diversity directly promotes the evolution of generalist parasites remains a debatable issue. Nevertheless, in an ongoing study (not included in this thesis) we analyzed the mean local host diversity against the mean local host specificity and we found a significant positive relationship between host diversity and host specificity, showing *Haemoproteus* communities are more generalists with increasing host diversity towards the equator. This promises extra support for our current hypotheses but requires further research. Along with host diversity the mode of parasite transmission acts directly on the host encounter rate and can thus create strong dilution effects (Civitello *et al.* 2015). The general phenomenon of the dilution effect is still fiercely debated (Salkeld, Padgett & Jones 2013; Civitello *et al.* 2015; McCallum 2015). However, a recent meta-analysis shows extensive prove that biodiversity generally decreases parasitism (Civitello *et al.* 2015). This was found for over 60 host-parasite systems and was independent of host density, study design and parasite specialization (Civitello *et al.* 2015). Future studies on the variation of host specificity of avipoxviruses and CRESS DNA viruses are needed, and could be assessed once we get an idea of their diversity and distributions throughout several bird communities around the world which differs in diversity. Our analyses with avipoxviruses showed extremely low prevalence of *Avipoxvirus* circulation in two megadiverse environments. Although mechanisms can be multiple, the dilution effect certainly proves part of the answer to understand why *Avipoxvirus* circulation is so low in these areas, although this should be confirmed on a bigger scale. We provide important data on blood parasite and *Avipoxvirus* communities to further investigate how the evolution of host specificity and dilution effects could be related. Finally, understanding this relationship and proving its generality are crucial for projecting disease outbreaks, which could impair species conservation and ecosystem services (Jones *et al.* 2008; Boyd *et al.* 2013; Civitello *et al.* 2015)

Historical processes and host specificity

Multiple historical processes influence the outcome of stable host-symbiont relationships between ecological communities and between different species in a community (Ostfeld & Keesing 2012). In a bird community in Ecuador we showed that the local parasite community is the result of multiple colonization events and that generalist parasites evolved repeatedly during their evolutionary history. In the same way, different hummingbird communities in Ecuador have parasite communities which evolved involving multiple host-switching and ecological fitting rather than strict co-speciation. This

corresponds with other studies which showed that host-switching is the major driver of species formation in avian blood parasites (Bensch *et al.* 2000; Ricklefs *et al.* 2004, 2014b; Hellgren *et al.* 2009). Species formation in avian blood parasites probably involves host expansion followed by host-parasite coevolution, resulting in local shifting of blood parasite lineages across host species over large taxonomic distances (Ricklefs *et al.* 2014b). The underlying mechanisms of this phenomenon are still poorly understood, especially the role of vector behavior (specialist vs. generalist), which needs to be addressed in future studies. Local *Avipoxvirus* communities seem to be assembled through similar processes as avian blood parasites. They follow the same pattern of symbiont community composition evolved from the import of phylogenetically distinct strains, instead of a result from local radiations. This is probably due to the fact that both symbionts are vector transmitted. Host-switching seems to be the rule for both symbiont models, which could be the result of the ecological filter acting on the probability of host switching. Since both parasites are transmitted by vectors with broad feeding patterns, the opportunities of host-switching are much higher than with parasites that are transmitted by contact (Poulin 2006). For example, in feather lice and bird associations, cospeciation seems to be the dominant process (Clayton & Moore 1997). Within this system fewer opportunities for host-switching exist. The opportunity of host-switching is thus one of the principal factors acting on the evolution of host parasite interactions on a macro- and micro evolutionary scale (Clayton, Bush & Johnson 2004; Poulin 2006). On the micro-evolutionary scale trade-offs between generalists and specialists seem to be important. As we have seen before, host diversity can act on the intensity of these trade-offs through dilution and amplification effects. Host specificity is mainly determined by the opportunities of colonization and the availability of suitable host species (Poulin 2006). Although the transmission mechanisms of CRESS DNA viruses are largely unknown, we found that closely related viruses of this group are found on hosts with very distantly related ancestries. Both CRESS DNA viruses found in this thesis are part of a virus clade which is found in insects, bats and marine environments. This suggests that CRESS DNA viruses have undergone major host switches during their evolutionary history. The rapid mutation and recombination rates of these viruses (Duffy *et al.* 2008; Martin *et al.* 2011) are probably key in understanding why host switching is so frequent in CRESS DNA viruses (Lefeuvre & Moriones 2015).

Interspecific diversity and host specificity

The host range of a parasite is equivalent to the realized niche of free living species, and whether niches are rather filled by generalists or specialists remains a critical question in ecology. We set out to explore this question by analyzing the evolution of host specificity in the spectacular radiation of hummingbirds to gain more insight if unique habitats are taken by generalist or specialist blood parasites. We analyzed if hummingbirds, which are characterized by a relative small body size, unique red blood cell features and a high metabolism (Suarez *et al.* 1991), harbor more specialist or generalist blood parasites. Surprisingly the hummingbird niche has been colonized by generalist *Haemoproteus*

parasites exclusively. Since hummingbirds have a singular physiology and metabolism, we expected to find specialist parasites which have adapted to this particular environment. Alternatively, if infection by these parasites is too costly for hummingbirds, we predicted to find hummingbirds hosting a community of generalist parasites, which may be the result from spillover infections from other birds. The latter was the case for three generalist parasites, but we found one generalist parasite (*Haemoproteus witti*) which depends heavily on hummingbirds in terms of prevalence and parasitaemia, compared to passerines where infection intensity and prevalence are much lower. This differential dependence on different host species raises the problem how to define specialization in this host parasite system. Is *Haemoproteus witti* more specialized in hummingbirds than in other birds? In order to tackle this question we will need to confirm if *H. witti* is successfully transmitted through passerines. Our findings open a debate of how to measure host specificity accurately in these parasites based on different detection methods. What we observe is that *H. witti* is a generalist by its PCR detection in many different species but as gametocytes are only visible in hummingbirds in a sense they are hummingbird specialists, challenging the current concept of host specialization in avian blood parasites. This has important implications in understanding the epidemiology and disease dynamics of blood parasites in wild bird populations.

Why did we not find greater parasite diversity in hummingbirds? Studies have shown that parasite richness is positively related to host body size (Gregory, Keymer & Harvey 1996). Perhaps parasite diversity and host specificity of the local parasite fauna are related. For example, there is a significant tendency for rich parasite faunas to include more specialists compared to species poor parasite faunas (Poulin 2006). We found a quite low diversity of hummingbird parasites which were all generalist, there may exist a negative relationship between parasite diversity and host specificity. A study analyzing the host-parasite network properties of freshwater fish and their metazoan parasites and mammalian hosts and their fleas showed specialist parasites tend to interact with hosts with high parasite richness, whereas hosts with low parasite richness tend to interact mainly with generalist parasites (Vazquez et al. 2005). This might provide an explanation of our observations in hummingbirds and our tropical bird communities and warrants further research on the architecture of host-parasite networks in tropical areas.

Host specificity and emergent infectious disease

Understanding the evolution and ecology of pathogen host specificity is vital to monitor potential disease outbreaks and to determine hotspots of potential emerging infectious disease (EID) (Woolhouse & Gowtage-Sequeria 2005; Keesing *et al.* 2010). Wildlife species can act as pathogen reservoirs which can form a threat to the conservation of biodiversity (Daszak *et al.* 2000). Our global analysis on the geographical patterns of blood parasite host specificity shows that the Neotropics is a hotspot for generalist *Haemoproteus* parasites in wild birds. Wild neotropical birds could be potential

reservoirs of generalist blood parasites, which are good candidates to colonize new habitats where they are accidentally introduced (Ewen *et al.* 2012). Although we found a very low incidence of *Avipoxvirus* lesions in our study areas, we demonstrated their generalist nature and their capacity to move along wide geographical areas which makes them important pathogens to monitor in the future. The expected size of an outbreak depends primarily on the number of introductions and the potential for transmission of a pathogen to a new host (Woolhouse, Haydon & Antia 2005). A recent study found that *Avipoxvirus* evolves at a rate of 2-8 x 10^{-5} substitutions/year and found their common ancestor on 10.000-30.000 years (Le Loc'h, Bertagnoli & Ducatez 2015). Although this estimate is based on three genes and requires deeper analyses, this suggests an extraordinary diversification capacity of these viruses on a short evolutionary timescale. Future studies on *Avipoxviru*s diversity and host specificity are crucial, especially in tropical areas where they received less attention. Finally, in our phylogenetic analysis of CRESS DNA viruses, we uncovered that closely related viruses often are found on a wide diversity of hosts and habitats, showing their extraordinary capacity of host switching. These results provide promising clues that CRESS DNA viruses are excellent models to study the variation in host specificity. Moreover, their high mutation and recombination rates could facilitate their ability to switch hosts and possibly cause new disease outbreaks (Rosario *et al.* 2012; Lefeuvre & Moriones 2015). Taken together, the knowledge of the global distribution of blood parasites, avipoxviruses and CRESS DNA viruses and the factors influencing their local success in different host environments will greatly contribute to our understanding of the risk of disease spread and emergence.

Conclusions

I. The evolution of generalist parasites has been promoted multiple times during the evolutionary history of *Plasmodium* and *Haemoproteus* in a megadiverse bird community in Ecuador. Moreover, the mean host specificity of the local *Haemoproteus* community was significantly higher than others outside the Neotropics. *Haemoproteus* parasite assemblages in Ecuador and South America are more generalist than elsewhere, making this continent a hotspot of generalist *Haemoproteus* parasites of wild birds.

II. In a tropical bird community in Ecuador, the generalist *Haemoproteus* parasites have a wide phylogenetic ancestry, they are closely related to specialist parasites and they are endemic to the Amazon forest, suggesting they evolved towards generalists upon colonization of this megadiverse region.

III. Hummingbirds are colonized by generalist *Haemoproteus* parasites which are all shared with other host orders which supports the idea that the unique blood physiology has not promoted parasite specialization (but see **V**). Instead this unique niche has been occupied by parasites which generally spill over from other sympatric bird species.

IV. Andean hummingbirds are infected with blood parasites belonging to various *Haemoproteus* clades with wide phylogenetic origins within the worldwide *Haemoproteus* diversity. This finding supports that multiple colonization events by *Haemoproteus* parasites have affected hummingbirds during their evolutionary history and shows that host-switching has been an important process in shaping the diversity of parasites in this unique family of birds.

V. *Haemoproteus witti* is a super generalist parasite infecting hummingbirds and other bird orders, but is more dependent on hummingbirds both in terms of prevalence and gametocymia compared to other bird species. This unveils hummingbirds as important reservoirs for this parasite in this region. The distinction in reservoir importance challenges the way we interpret host specialization in blood parasites and other host parasite systems, and requires the use of multiple parasite detection techniques to reveal true patterns of host specificity.

VI. Bird communities in Ecuador and French Guiana show an extremely low prevalence and diversity of *Avipoxvirus* lesions affecting local bird species. Two new strains were found at different altitudes along an elevational gradient in Ecuador while no strains were detected in the lowland Amazon of French Guiana. Both strains have distant evolutionary trajectories with one strain placed in an American clade of Axipoxviruses while the second one is closely related to a strain found in the Madeira Archipelago. These results reflect the low circulation and diversity of *Avipoxvirus* lesions in tropical bird communities, and require future investigations to confirm this as a global phenomenon.

VII. Avipoxviruses are usually very generalist and they are widely distributed over continents. These two attributes help us to understand why avipoxviruses are good candidates to emerge under favorable ecological and evolutionary conditions, as has occurred multiple times in the past.

VIII. Two novel CRESS DNA viruses are described on an *Avipoxvirus* lesion of a common bird species in Ecuador. This discovery expands the current knowledge of these viruses about their potential hosts, distributions and molecular organization. Extensive host switching has taken place during the evolutionary history of these viruses between hosts and habitats of with very diverse origins.

Future Research

Measuring host specificity

In this thesis we challenged the current concept of host specialization in avian blood parasites. We showed that host specificity could be interpreted differently based on the use of different detection techniques. *Haemoproteus witti* was shown to be much more generalist when molecular techniques were considered compared to microscopical detection. In order to be more certain about parasite host specificity we recommend using both detection techniques when classifying host generalist versus host specialists. This will be very important in future studies on avian blood parasites, especially when host specificity patterns are analyzed. Secondly, throughout the thesis we have been using the S^*_{TD} index (Hellgren *et al.* 2009), which has proven to be a good index to quantify host specificity. Nevertheless, it does not consider prevalence data and it only includes taxonomic levels between different host species, without taking in account bird phylogeny. We feel it is opportune to work on an improved version of this index which includes these two important parameters for future studies.

Host parasite networks

Is host specificity a species character? If variation in host specificity for one parasite in different populations is less pronounced than the variation in host specificity for different parasite species we could conclude that host range is a species trait. For this purpose we will analyze whether parasite lineages show the same host specificity in different bird communities on a worldwide scale. We will use our data plus the MalAvi database. We will construct host-parasite networks for all local communities and calculate host specificity, nestedness, modularity and connectedness of multiple networks containing the same parasite lineage. As such we will gain insights if a particular parasite lineage shows the same host specificity in different communities around the world on global scale. This will allow to understand what parasites do over a large range of biotic and abiotic conditions which is closely equivalent to their realized niches (Morand, Krasnov & Littlewood 2015). Secondly we will explore the host specificity of one particular lineage on our elevational and bird diversity gradient in Southeast Ecuador. This approach will give us a local perspective on how host specificity of one lineage may be stable or may vary throughout several bird communities. The latter reflects what parasites do locally which represents their truncated niches, which can be very distinct to their realized niches (Morand *et al.* 2015).

Hummingbirds as models for avian malaria studies

Hummingbirds are a fascinating group of animals which diversified among a wide variety of ecosystems in the Neotropics. Although they are well studied, little is known about the effects of blood parasites (*Haemoproteus*, *Plasmodium*, *Leucocytozoon*) on their physiology, survival and

behavior. In the future, we aim to study the consequences of blood parasite infection on the feeding behavior of Amazonian understory hummingbirds in the Nouragues reserve, French Guiana. By means of experiments we will explore if parasite infection influences the time a hummingbird spends at a specific resource, the hummingbird's capacity to visit several resources under infection, and the resting time between feeding bouts compared to healthy individuals. We will focus our study on *Phaethornis*, *Campylopterus* and *Thalurania* species, which are the most common hummingbirds thriving around *Heliconia* patches at the Nouragues reserve. When possible, other hummingbird species will be included if captured in sufficient numbers. Birds will be captured with mist nets and will be released in an experimental enclosure where different hummingbird feeders will be monitored with cameras. After each feeding trial, a small blood sample will be taken to probe their status of infection and the birds will be released unharmed at the site of capture. The results will show us how malaria infection can influence the feeding behavior of important pollinators in the rainforest and how ecosystem services like pollination can indirectly be affected by blood parasites.

Feather mite host specificity and diversity

During this PhD project we collected feather mites of 220 species of tropical birds. For our Ecuadorian samples we managed to mount 10 specimens on glass slides per host species and have determined them to the genus level. Since these are preliminary results, we aim to progress on the identification of these mites to the species level, which will involve the description of many new species for science. With the help of Dr. Sergei Mironov we already described a new feather mite species named *Piciformobia cinnycerthiae* collected from the Rufous Antwren (*Cinnycerthia unirufa*) in the Podocarpus National Park. Once all the sampled mites from the database are identified, we can start to design primers to barcode different mite species. Moreover we will look for genetic markers to infer phylogenetic relationships, which will be useful to understand the evolution of these fascinating symbionts. We already characterized the feather mite fauna for hummingbirds, which consists of several mite genera unique for hummingbirds. We collected data on the distribution of these feather mites along the wing, which will provide clues on the forces acting on feather mite niche filling.

Avipoxvirus circulation in tropical bird communities

At the start of this PhD project we expected to find many more *Avipoxvirus* in the two studied bird communities in the tropics. We collected data from 943 birds in Ecuador and 307 birds in French Guiana, where we only detected three lesions in Ecuador. This result was very interesting but challenging to present with a global perspective. *Avipoxvirus* prevalence is low in tropical wild bird communities and difficult to study, especially because it is hard to get representative sample sizes. We found no *Avipoxvirus* in our Guianan sample, which motivated us to return for a 2 month expedition in order to gather more data. We captured an additional 583 birds which sums up to 890 birds in total for this study site. Interestingly we detected a possible *Avipoxvirus* in a fulvous-crested tanager

(*Tachyphonus surinamus*), which needs to be confirmed by molecular analyses soon. Along with our sample from Ecuador, we have evidence that tanager species (family Thraupidae) could be reservoir species of *Avipoxvirus*. This makes them good candidates to study *Avipoxvirus* prevalence at the intraspecific level in the tropics.

CRESS DNA virus diversity and Host specificity

Originally the thesis would focus on *Avipoxvirus* and *Papillomavirus* infections of wild birds. By studying an *Avipoxvirus* lesion of a bird in Ecuador we suddenly detected two circular virus genomes belonging to the group of CRESS DNA viruses. In the last decade, the discovery of CRESS has risen spectacularly, both from human-made environments and natural environments. We are now developing universal primers for the detection of CRESS in bird samples. We just returned from an expedition from the Nouragues Reserve in French Guiana, where we collected throat and cloaca swabs stocked in virus medium to explore the diversity and evolutionary relationships of these viruses in bird communities of the Amazon rainforest. We further want to analyze the network interactions of CRESS DNA viruses and their hosts and how these virus communities are shaped.

Vector research

It has become clear that in order to understand the evolution of host specificity in blood parasites one must include studies on the diversity and host specificity of vectors. In the following years, we will collect vectors on all sampling locations to gain insights on their identity and diversity. Furthermore, we will try to point out the putative vectors for the most generalist parasites (*H. witti*). This will be done by controlled experiments and dissections and extractions of the vector salivary glands, in order to prove the successful transmission of the parasite into the vertebrate host.

White-plumed Antbird

(*Pithys albifrons*)

Chapter 1: Discovering potential sources of emerging pathogens: South America is a reservoir of generalist avian blood parasites.

This chapter is based on the manuscript: **Moens M.A.J.** & Pérez-Tris J. 2016. Discovering potential sources of emerging pathogens: South America is a reservoir of generalist avian blood parasites. *International Journal for Parasitology*.46:41-49.

Discovering potential sources of emerging pathogens: South America is a reservoir of generalist avian blood parasites.

Michaël A. J. Moens & Javier Pérez-Tris

Abstract

Generalist pathogens are capable of infecting a wide range of host species, and may pose serious disease emergence threats if accidentally moved outside their native areas. So far little effort has been devoted to identify geographic areas that may act as reservoirs of generalist pathogens. According to current theory, where host diversity is high, parasite specialization in one host species may be penalized by reduced host availability, while generalist parasites may benefit from the exploitation of various host species. Therefore natural selection could favor generalist parasites where host diversity is high. Here we explored if in a highly diverse bird community in Ecuador a generalist strategy is promoted among local *Haemoproteus* and *Plasmodium* blood parasites compared to similar parasite communities throughout the world. We reconstructed the phylogenetic relationships of every parasite lineage in order to understand the evolution of host specificity in this megadiverse area. We found high levels of host generalization for both parasite genera, and the mean host range of the *Haemoproteus* community in Ecuador was significantly higher than other parasite communities in other areas outside the Neotropics. Generalist *Haemoproteus* parasites in this bird community had diverse phylogenetic ancestry, were closely related to specialist parasites and were apparently endemic to the Amazon showing that different parasites have independently evolved into host generalists in this region. Finally we show that *Haemoproteus* communities in Ecuador and South America are more generalist than in temperate areas, making this continent a hotspot of generalist *Haemoproteus* parasites for wild birds.

Key words*:* avian malaria, dilution effect, Ecuador, *Haemoproteus*, host specificity, *Plasmodium*.

Introduction

Understanding the evolution and ecology of generalist parasites is crucial to prevent disease outbreaks and to determine hotspots of potential emerging infectious disease (EID) (Woolhouse and Gowtage-Sequeria, 2005; Keesing et al., 2010) . Different wildlife species can act as pathogen reservoirs which can form a threat to domestic animals and humans and pose a significant hazard to the conservation of biodiversity (Daszak *et al.* 2000). A global analysis on the geographical patterns of EID emergence shows that vector-borne diseases and zoonotic pathogens with wildlife origins are more concentrated at lower latitudes, but surveillance efforts have mainly concentrated on temperate areas (Jones *et al.* 2008). In contrast, a recent study on rodent reservoir species for future zoonotic disease outbreaks found that hotspots are mainly located in temperate areas (Han *et al.* 2015). For the emergence of zoonotic EID's with a wildlife origin, wildlife host species richness is a significant predictor (Jones *et al.* 2008). Therefore it is especially important to monitor tropical areas and identify hotspots of potential EID before their local and large-scale emergence.

The host range of a parasite evolves through natural selection and is determined by different ecological and physiological factors (Combes 2001). The opportunity of finding the correct hosts is one of the most important ecological factors shaping host specificity (Combes 2001; Schmid-Hempel 2011). When a host species is abundant, specialist parasites have an advantage over generalist parasites if specialization favors host monopolization. However, if the relative abundance of different host species is low, as usually happens in highly diverse environments, parasite transmission among hosts of the same species can decrease (the dilution effect; Keesing et al., 2006)). In these circumstances, generalist parasites may compensate for reduced compatibility to a single host species if broadening host range increases the total host encounter rate and disease transmission (the amplification effect; Keesing et al., 2006)). Therefore, in highly diverse host communities a generalist strategy of host exploitation may be promoted among local parasite species, making them important areas to monitor for potential disease outbreak.

Avian haemosporidian parasites are a diverse group of parasites which infect most bird species worldwide (Valkiūnas, 2005; Bensch et al., 2009) and are easily moved with host introductions where they already have caused disease emergence problems (van Riper III et al., 1986; Schoener et al., 2014). *Haemoproteus* and *Plasmodium* parasites are protozoans that infect red blood cells and organs of birds and are transmitted by biting midges and mosquitoes respectively (Valkiūnas 2005). The MalAvi database (Bensch *et al.* 2009) offers a unique opportunity to study the variation in host specificity in detail as it holds information of more than 1900 unique lineages of avian blood parasites across the world collected from approximately 300 publications. We have a good knowledge of the degree of host specialization of these parasites, which is usually higher for *Haemoproteus* than for *Plasmodium* (Ricklefs and Fallon 2002; Beadell et al., 2009; Hellgren et al., 2009).

Host specialization has been proven important in determining invasion success through host switches undergone by introduced blood parasites (Ewen *et al.* 2012). Generalist blood parasites of the genera *Plasmodium* and *Haemoproteus* are a threat to birds worldwide, occasionally causing significant mortalities in native bird species (Atkinson & Lapointe 2009). For example, the introduction of *Plasmodium relictum* and its main vector *Culex quinquefasciatus* to the Hawaii archipelago triggered a dramatic decline and extinction of native species of honeycreepers (van Riper III *et al.* 1986). Likewise, generalist *Haemoproteus* parasites have caused mortality in various species of captive parrots throughout Germany (Olias *et al.* 2011). It has been shown that generalist *Plasmodium* and *Haemoproteus* parasites are better colonizers of island avifauna (Ewen *et al.* 2012; Pérez-Rodríguez *et al.* 2013b) compared to specialist parasites and that they can reach the highest prevalence in a single host species, making them the most common in the host community (Hellgren et al., 2009). Generalist blood parasites are good candidates to develop into emerging disease risks when moved outside their native areas. Therefore, it is important to determine which geographic areas of the world house the greatest diversity of these potential sources of emerging infectious diseases.

Here we analyzed the host specificity of avian *Haemoproteus* and *Plasmodium* blood parasites in a megadiverse bird community of Wisui, located in a lowland tropical forest in Southeast Ecuador and compare it with other areas throughout tropical and temperate areas. Ecuador forms part of the tropical Andes biodiversity hotspot (Myers *et al.* 2000) and its 1628 described avian species make it one of the most bird diverse countries in the world (Ridgely & Greenfield 2006) and hence a good candidate to explore if high bird diversity promotes the evolution of generalist parasites. First, we analyzed whether *Haemoproteus* parasites have evolved a host generalist strategy in the diverse host community of Wisui as natural selection could favor this strategy through the benefits of the amplification effect. As *Haemoproteus* parasites are normally more host specific, they are a good model to explore this hypothesis. In these circumstances, *Plasmodium* parasites are expected to be generalists as well, as they usually are everywhere in the world. Secondly, if the parasite communities of Wisui are generalist, we explored if these parasites have diverse phylogenetic origins and are related with specialist parasites, which would support the hypothesis that they evolved towards generalism upon colonization. There are two possible evolutionary trajectories leading to the evolution of a generalist community of blood parasites. The parasite community could represent a local radiation of generalist parasites formed by the descendants of a generalist ancestor. Alternatively, the parasites could be distantly related to one another, sustaining the hypothesis of multiple origins of the local parasite fauna. Finally we compared the host specificity of this parasite fauna with similar *Plasmodium* and *Haemoproteus* communities throughout tropical and temperate areas to test if Ecuadorian parasite communities are more generalist than elsewhere. This study will contribute to our knowledge of how generalist parasites evolve and help us identify reservoirs of potential EID caused by avian blood parasites.

Materials and Methods

Study area and field methods

The study was conducted in the Wisui reserve on the west slopes of the Cutucú Mountain range in Southeast Ecuador (Taisha canton, Morona-Santiago Province). The range is classified as an Important Bird Area with more than 480 registered bird species (Santander *et al.* 2009), classifying it as a megadiverse biodiversity hotspot (Myers *et al.* 2000). Our sampling area (the Wisui forest: 02°07'S, 77°44'W, 650 m altitude) is located within a 3000-ha primary forest. The climate conditions are tropical and very humid, with an annual average temperature between 23 and 25.5 °C and an average precipitation of 3000 mm. During the sampling period (April 2010), the weather was cloudy with frequent heavy rainfalls.

We set up 14 mist nets (12 m long × 2.5 m high, 25 mm eye) during 30 days at four different sites in the rainforest, which were similar in vegetation structure and separated by less than 500 m. We determined species of all captured birds by consulting several references (Hilty & Brown, 1986; Ridgely & Greenfield, 2006; Schulenberg *et al.*, 2007) and according to Remsen *et al.* (2014). We took standard body measurements of all captured birds (wing, tail and tarsus length, and body mass), and photographed individuals to confirm difficult species identifications. We collected blood samples (5-80 µl, depending on body size) by puncture of the brachial vein. We used part of the blood to make two blood smears, which were air dried and fixed in absolute ethanol. The remaining blood was kept in absolute ethanol to preserve DNA, at ambient temperature in the field and at -20 ºC until molecular analysis. Once processed, birds where marked using an individual feather clip code to avoid repetition, and released unharmed at the site of capture.

Laboratory methods

Blood smears were stained with GIEMSA solution (pH 7.2) for 75 min. Each blood smear was observed with a light microscope (LEICA DM2500). We first scanned smears at relatively low magnification (400×) to cover the whole smear in the search for parasites. Then we screened at 1000× focusing on intra-erythrocytic parasites, until ca. 100.000 erythrocytes were inspected.

We extracted total DNA from blood samples with a standard ammonium acetate protocol. We amplified the MalAvi barcode for avian haemosporidians (479 bp of the cytochrome *b* gene; (Bensch *et al.* 2009)), which has been sequenced for the majority of the known diversity of these parasites and consequently facilitates the analysis of their evolutionary relationships. We made sure that all samples were of good quality for PCR by amplifying bird sexing markers (Fridolfsson & Ellegren 1999). We screened for parasite infections using the nested PCR protocol developed by Waldenström *et al.*(2004), which was specifically designed to amplify *Haemoproteus* and *Plasmodium* DNA. The first

PCR, in a total volume of 25 µl, included 25 ng of total genomic DNA, 1.25 mM of each dNTP, 1.5 mM MgCl2, 10 µM of the primers HaemNF and HaemNR2 and 0.5 units AmpliTaq DNA polymerase (Applied Biosystems). We used the following reaction conditions: a denaturation step of 94 °C for 3 min, 20 amplification cycles of 30 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C, and a final elongation step for 10 min at 72 °C. We used 1 µl of the PCR product as the template for the second PCR, with primers HaemF and HaemR2 and the same reaction conditions, except for using 35 amplification cycles (Waldenström *et al.* 2004). We visualized 4 µl of the final PCR product on a 2% agarose gel stained with ethidium bromide. Positive and negative controls were included in each PCR batch. PCR products were precipitated by adding 11 µl of 8M NH4Ac and 33 µl absolute ethanol, and resuspended in 15-20 µl water. We sequenced them from both ends (using primers HaemF and HaemR2) with a dye-terminator AmpliCycle sequencing kit and an ABI PRISM^{TM}3700 sequencing robot (Applied Biosystems).

Phylogenetic analyses

The sequences were manually aligned and edited using BioEdit (Hall 1999). Sequences differing by one nucleotide substitution were considered to represent unique lineages (Bensch et al., 2004, 2009). We identified parasite lineages by means of local BLAST analyses of the MalAvi database (Bensch *et al.* 2009) and Genbank. The new lineages were coded following the nomenclature of the MalAvi database (Bensch *et al.* 2009). The data from Wisui were sent to MalAvi and all new sequences were uploaded to GenBank (accession numbers KT373858-KT373878, supplementary table S1).

We reconstructed the phylogenies of *Haemoproteus* and *Plasmodium* parasites separately including the parasite lineages found in Wisui and those parasites for which complete MalAvi sequences (479 bp amplified with primers HaemF and HaemR2) were available from the MalAvi database (Bensch *et al.* 2009) for a total of 303 *Haemoproteus* and 223 *Plasmodium* lineages. Many lineages in MalAvi lack data for many base pairs which could possibly bias the phylogeny's outcome. Moreover, we wanted to create a phylogeny with the most accurate data available for these parasites. Thus, lineages with an incomplete MalAvi barcode were not included in these analyses. We inferred a Bayesian analysis with the BEAST 2.0 -software (Bouckaert *et al.* 2014) for each parasite genus, using the most appropriate substitution model according to the Bayesian Information Criterion implemented in MEGA 5.2 (Tamura *et al.* 2011): GTR + G + I for both *Haemoproteus* and *Plasmodium*. We specified the parameters for the BEAST-run in BEAUTI 2.0 (Bouckaert *et al.* 2014) and MCMCs were run for 10^9 generations, sampling every 100.000 trees. A 25% burn-in was removed from the analysis. A strict clock model and a Yule speciation prior were used. Traces were inspected for convergence with Tracer 1.5 (Rambaut & Drummond 2007). The 10000 resulting trees were summarized with TreeAnnotator v2.1.2 (Rambaut & Drummond 2007) and the phylogenies with the posterior probabilities of the nodes were displayed in Mesquite 2.75 (Maddison & Maddison 2011) and FigTree

v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) for further analysis. The *Haemoproteus* tree was manually rooted with various lineages of *Haemoproteus* infecting columbiform species and four species of *Leucocytozoon* were used to root the *Plasmodium* tree.

Host range analysis

We compared the mean host range of the *Haemoproteus* and *Plasmodium* communities found in Wisui with parasite communities sampled throughout the world. To this end, we first calculated a host range index for each parasite lineage found in this study and in other studies worldwide. We computed the S^*_{TD} index (Hellgren *et al.* 2009), which measures a host range for each parasite considering the diversity of host species, the taxonomic distance between host species and its variance (Poulin & Mouillot 2003; Hellgren *et al.* 2009). The S^*_{TD} index was calculated as follows:

$$S^*_{TD} = S_{TD} + \frac{s-1}{1 + VarS_{TD}{}^{s}}$$

$$S_{TD} = 2\frac{\sum\sum_{i<j}\omega_{ij}}{s(s-1)}$$

$$VarS_{TD} = \frac{\sum\sum_{i\neq j}\left(\omega_{ij} - S_{TD}\right)^2}{s(s-1)}$$

where ω_{ij} is the taxonomic distance between host species *i* and *j* (i.e., how many taxonomical steps need to be taken to get to their most recent common ancestor) and *s* is the number of host species infected by the parasite (Hellgren *et al.* 2009). We define a generalist as a parasite that is found in two or more unique host species. A specialist is a parasite found in one unique host species for at least two times. Generalist parasites score a S^*_{TD} value higher than 2 while specialist are assigned the value zero (as the divisor in the formula adds up to zero when s=1).

We compared the average local S^*_{TD} values obtained for *Haemoproteus* and *Plasmodium* parasite assemblages found in Wisui with those computed for other localities worldwide, which were obtained from a review of 27 different studies in which avian haemosporidian parasites had been investigated at the bird community level (Supplementary data S1). These studies were selected based on literature research on the ISI Web of Science database (key words: *Haemoproteus*, *Plasmodium*, avian malaria, access date: 8th of June 2015) and the MalAvi database (access date: 8th of June 2015) with the number of parasite cyt. *b* lineages of each parasite genus encountered as selection criterion for the comparative analyses (n >= 5 for *Haemoproteus*, n >= 8 for *Plasmodium*: based on the parasite diversity found in our study, see results and Supplementary data and table S1). Singleton lineages (those appearing only once in the sample and therefore necessarily scoring single hosts) cannot be named specialist lineages as more sampling could increase their host range. Therefore, we excluded

these observations from the analysis as they are likely to bias the mean host specificity values towards higher host specificity (however, a less conservative analysis that included all data led to the same conclusions). To be able to make a comparison on a global scale the best marker to use is the one from MalAvi, as the great majority of studies are based on this part of the cytochrome *b* region. We also added studies where a different part of the mitochondrial genome was amplified to be able to make additional comparisons in South America. The studies included in our final dataset involved 28 parasite assemblages for *Haemoproteus* and 21 for *Plasmodium*, from a total of 30 different locations (12 of them with data for both genera). These studies sampled a mean (± SE) diversity of 21.6 ± 14.6 and 23.2 ± 23.1 lineages of *Haemoproteus* and *Plasmodium*, respectively. We used these studies to examine how likely it is to find a parasite community with a mean host range equal or higher than the observed in Wisui to determine if we indeed have more generalist parasites in this geographical area. To this end, we generated 1000 random assemblages of 8 *Plasmodium* or 5 *Haemoproteus* lineages from the diversity of parasites found in each study, and used the proportion of assemblages with local S^*_{TD} values equal or higher than the observed in Wisui as a significance value for differences between Wisui and the corresponding community.

Finally, to examine if the parasites in Wisui share close ancestry with specialist or generalist parasites around the world we traced the evolution of host specificity over the phylogenetic trees that we built for *Haemoproteus* and *Plasmodium*. We calculated a global S^*_{TD} value for each lineage based on all infections in MalAvi and we reconstructed the ancestral states of host specificity through the phylogeny using the trace character history option (parsimony ancestral state method) in Mesquite 2.75 (Maddison & Maddison 2011). For a better visualization of the resulting tree we set the global S^*_{TD} values for host specificity in 5 categories, namely parasites infecting only one host species (1), various species of the same genus (2), or species of different genera (3), families (4) or orders (5).

Results

Parasite lineage diversity and phylogenetic relationships

We analyzed 345 birds from Wisui, belonging to 89 species of 21 families (Supplementary table S1). Of these, 56 tested positive (both by microscopy and PCR), and cyt. *b* sequences were obtained for 54 infections. Eight *Haemoproteus* lineages were retrieved from 17 bird species of 8 families, and 13 *Plasmodium* lineages were found among 17 species of 5 families (Supplementary Table S1).
Chromatograms were analyzed in detail and showed no sign of double infections (Pérez-Tris & Bensch 2005).

According to MalAvi (Bensch *et al.* 2009) and Genbank databases, 15 of the 21 parasite lineages found in Wisui were new (Access date: 3th of August 2015; Supplementary Table S1). The lineage PACPEC02 (Genbank: JX029911) was already detected in two other studies, one in Brazil (Lacorte *et*

al. 2013) and one in Papua New Guinea (Beadell *et al.* 2004). TANSCH01 was detected in Costa Rica (Archer in Genbank: JN819385) and at the San Diego Zoo (Schrenzel *et al.* 2003) in the same host species as in Wisui (*Tangara schrankii*). MYRMAX01 was also found in Peru (Witt & McNew in Genbank: JQ988606) and LEPCOR04 in Costa Rica (Archer in Genbank: JN819332), again in the same host species as in this study (*Myrmotherula maxillaris* and *Lepidothrix coronata*, respectively). Finally LEPCOR01 and TACRUB01 were detected in Peru in (Marzal in Genbank: KF482355; KF482356) in *L. coronata* and *Tachuris rubrigastra* respectively. Both *Haemoproteus* and *Plasmodium* lineages found in this study showed a wide phylogenetic distribution throughout the known lineage diversity of each genus, although the latter genus clustered in two clades (Fig. 1).

Host range analysis

Of the 21 parasite lineages found in Wisui, 10 were generalists, 3 were specialists and 8 were only recorded once in one host species. The latter (singletons) were excluded from further analyses. The *Haemoproteus* lineage LEPCOR03 was the most generalist parasite (S^*_{TD} = 8.47), infecting 7 host species of two families (Supplementary Table S1). The *Haemoproteus* community from Wisui was significantly more generalist (S^*_{TD} = 3.495) than other 15 of 21 communities sampled outside of South America (average mean S^*_{TD} = 1.29 ± 0.98) (Fig. 2, Table 2). Within South America, the host specificity of *Haemoproteus* was not significantly different in Wisui compared to the 7 other communities, showing South America harbors more generalist *Haemoproteus* parasites than elsewhere.

The mean host range of *Plasmodium* parasites from Wisui (S^*_{TD} = 3.77) was high compared to the average of other bird communities worldwide (average S^*_{TD} =1.55 ± 0.86; Table 1). Specifically, 11 out of 20 communities with 8 or more *Plasmodium* lineages scored significantly lower S^*_{TD} values than Wisui and 4 of these were located in South America (Fig. 2, Table 1). There was no apparent geographical pattern in the variation of host specificity for this genus. Out of 9 sites where both genera could be studied, only 2 had *Haemoproteus* communities that were more generalist on average than *Plasmodium* communities and these were again South American.

The quantitative reconstruction of the ancestral states of host specificity agreed in placing all the generalist *Haemoproteus* lineages (LEPCOR01, EUXAN01, TANSCH01, PACPEC02) in close ancestry with specialist parasites or evolved from a specialist ancestor (Fig.1). The generalist *Plasmodium* parasites (HYLSUB01, LEPCOR02, MYRMAX01, MYRMYO01, THAMAE01, THACAE01) evolved from generalist ancestors, and share their most common ancestor with specialist parasite lineages from other Neotropical and temperate areas (Fig. 1).

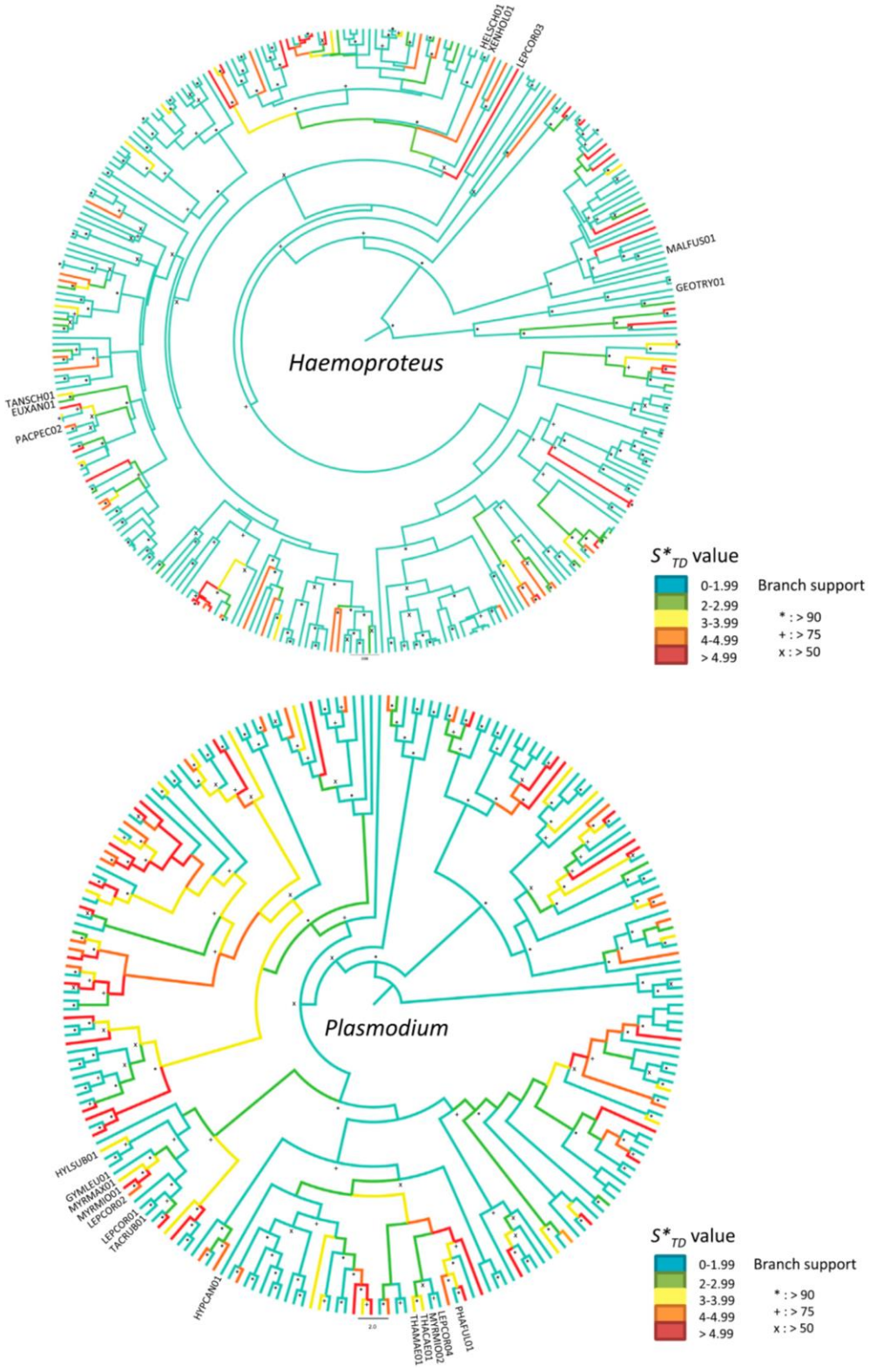


Figure 1. Phylogenetic relationships of 303 *Haemoproteus* cyt. *b* lineages and 223 *Plasmodium* cyt. *b* lineages found in Wisui and worldwide inferred with Bayesian analyses. The evolutionary history of host specificity of *Haemoproteus* and *Plasmodium* was calculated with a quantitative approach, in which each branch tip scores the global S^*_{TD} value (host-specificity index) of its lineage. For simplicity, S^*_{TD} values have been divided into 5 categories: 0-1.99 representing parasites infecting a single host species (blue branches); 2-2.99 (2 host species of the same genus, green); 3-3.99 (2 host genera, yellow); 4-4.99 (2 host families, orange); >4.99 (2 or more host orders, red). Parasites found in Wisui are labelled. The posterior probabilities for branch support are marked with the following signs according to their value: *(>90), x (>75), + (>50).

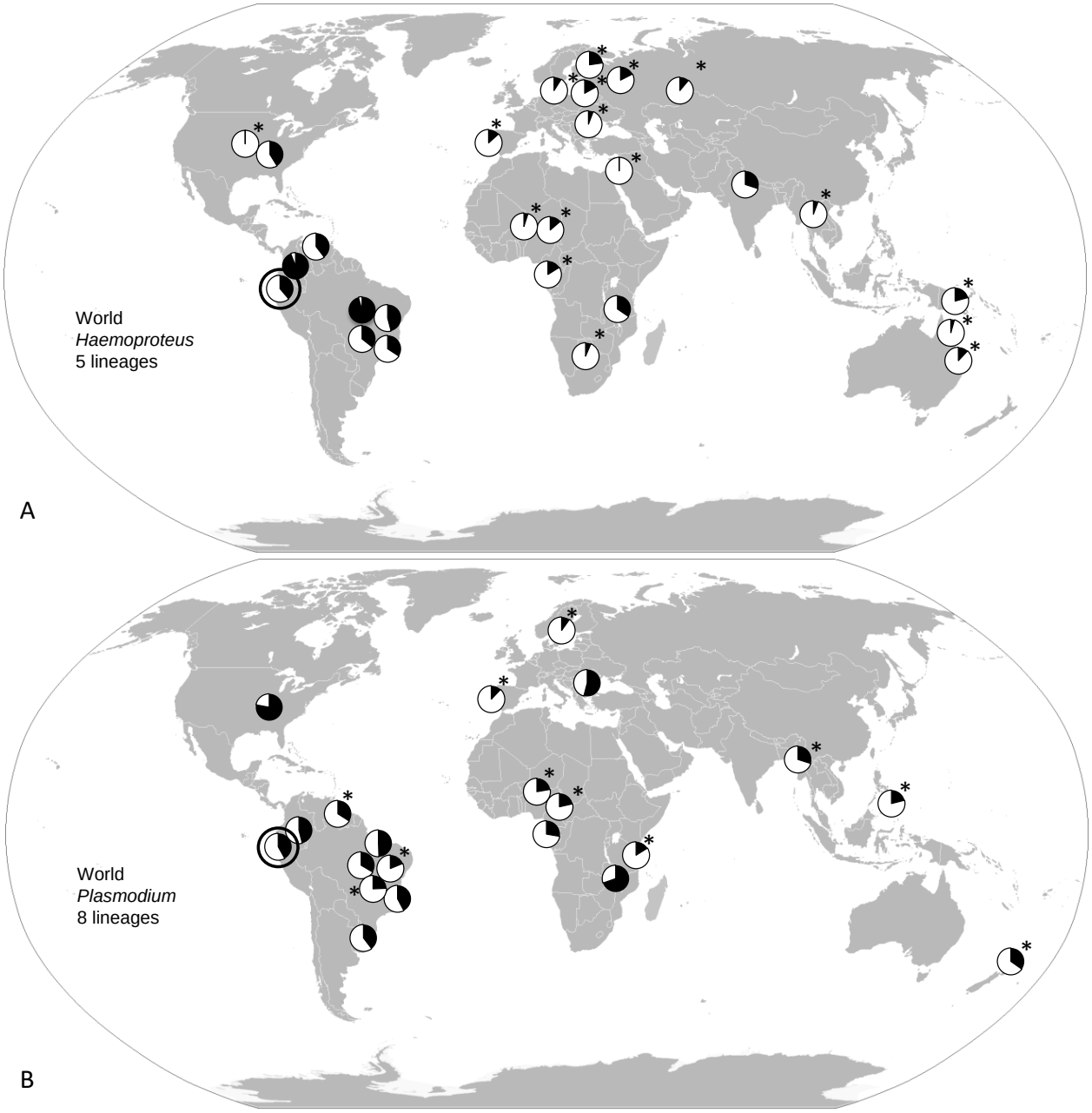


Figure 2. Variation in the mean host range of randomly assembled communities of *Haemoproteus* (5 lineages) and *Plasmodium* (8 lineages) in South America and the world, as obtained from sampling sites where local S^*_{TD} indexes (mean host specificity value) could be computed based on published data. In all graphs, the host range of the parasite community is shown in black, assuming a full circle represents $S^*_{TD} = 9$. Statistically significant differences between Wisui (encircled) and other sites are marked with stars.

Table 1. Mean S^*_{TD} values (host specificity index) and their standard deviations (SD) for 21 parasite communities of the world that included at least 8 *Plasmodium* lineages which were recorded more than once. For each site, 1000 communities of 8 lineages were randomly generated from the local diversity of parasites. The proportion of simulated parasite communities that scored an average S^*_{TD} value equal or higher than the one obtained for Wisui was used to test for the statistical significance of the comparison between Wisui and each site. The mean S^*_{TD} indexes of the simulations and significant p-values are highlighted in bold.

Nr.	Locality	Continent	Nr. of lineages	Nr. of Singletons	Mean S^*_{TD}	SD	8 *Plasmodium* S^*_{TD}	SD	p-value	Marker	Reference
1	Gabon	Africa	35	10	2.50	3.45	**2.49**	0.98	0.12	Malavi	Beadell et al.,2009
2	Cameroon	Africa	16	2	1.79	1.63	**1.93**	0.35	**<0.001**	Malavi	Loiseau et al.,2012b
3	Tanzania	Africa	11	3	1.45	4.10	**1.45**		**<0.001**	Malavi	Loiseau et al.,2012b
4	Malawi	Africa	82	52	6.22	7.46	**6.27**	2.21	0.88	Malavi	Lutz et al.,2015
5	Myanmar	Asia	20	6	2.66	2.61	**2.69**	0.61	**0.03**	Malavi	Ishtiaq et al.,2007
6	New Zealand	Australasia	9	1	3.13	3.09	**3.13**		**<0.001**	Malavi	Ewen et al.,2012
7	Philippines	Australasia	9	7	2.00	2.83	**1.89**	0.43	**<0.001**	Malavi	Silva-Iturizza et al.,2012
8	Sweden	Europe	13	4	0.89	1.83	**0.89**	0.22	**<0.001**	Malavi	Hellgren et al.,2007b
9	Bulgaria	Europe	14	1	4.86	3.51	**4.86**		1.00	Malavi	Dimitrov et al.,2010
10	Portugal	Europe	15	2	1.46	2.51	**1.09**		**<0.001**	Malavi	Ventim et al.,2012
11	USA	North America	13	0	7.02	2.40	**7.02**		1.00	543 F- 926R	Ricklefs et al.,2005
12	Guyana	South America	15	7	3.06	2.00	**3.06**		**<0.001**	Malavi	Durrant et al.,2006
13	Uruguay	South America	13	3	3.55	3.34	**3.57**	0.53	0.39	Malavi	Durrant et al.,2006
14	Brazil	South America	11	2	2.48	2.82	**4.45**	0.54	0.78	343F-496R	Belo et al.,2011
15	Brazil	South America	97	53	3.90	3.59	**3.01**	1.19	0.25	Malavi	Lacorte et al.,2013
16	Brazil	South America	10	3	2.43	2.30	**3.81**	0.23	1.00	Malavi	Lacorte et al.,2013
17	Brazil	South America	21	8	1.69	2.29	**2.19**	0.38	**<0.001**	Malavi	Lacorte et al.,2013
18	Brazil	South America	20	6	1.21	1.53	**1.69**	0.25	**<0.001**	Malavi	Lacorte et al.,2013
19	Brazil	South America	22	7	1.24	1.90	**1.69**	0.37	**<0.001**	Malavi	Lacorte et al.,2013
20	Ecuador	South America	30	6	4.17	5.13	**4.14**	1.48	0.57	3932F-3932R	Svensson-Coelho et al.,2013
21	Ecuador	South America	13	5	3.77	1.27				Malavi	This study

Table 2. Mean S^*_{TD} value (host specificity index) and their standard deviations (SD) for 28 parasite communities of the world that included at least 5 *Haemoproteus* lineages which were recovered more than once. For each site, 1000 communities of 5 parasites were randomly generated from the local diversity of parasites. The proportion of simulated parasite communities that scored an average S^*_{TD} value equal or higher than the one obtained for Wisui was used to test for the statistical significance of the comparison between Wisui and each site. The mean S^*_{TD} indexes of the simulations and significant p-values are highlighted in bold.

Nr.	Locality	Continent	Nr. of lineages	Nr. of Singletons	Mean S^*_{TD}	SD	5 *Haemoproteus* S^*_{TD}	SD	p-value	Marker	Reference
1	Nigeria	Africa	20	6	0.64	1.08	**0.44**	0.37	**<0.001**	Malavi	Waldenström et al.,2002
2	Gabon	Africa	30	8	1.47	2.16	**1.45**	0.84	**0.01**	Malavi	Beadell et al.,2009
3	Nigeria	Africa	55	27	1.21	1.66	**1.23**	0.67	**<0.001**	Malavi	Hellgren et al.,2007b
4	Botswana	Africa	9	4	0.92	1.50	**0.58**	0.27	**<0.001**	Malavi	Ishtiaq et al.,2012
5	Malawi	Africa	69	38	3.02	2.67	**3.10**	1.07	0.35	Malavi	Lutz et al.,2015
6	Myanmar	Asia	20	15	0.60	1.34	**0.51**	0.22	**<0.001**	Malavi	Ishtiaq et al.,2013
7	India	Asia	16	5	2.74	2.25	**2.71**	0.76	0.17	Malavi	Ishtiaq et al.,2007
8	Russia	Asia	12	3	1.22	1.48	**1.00**	0.47	**<0.001**	Malavi	Palinauskas et al.,2013
9	Australia	Australasia	26	11	0.40	0.83	**0.39**	0.30	**<0.001**	Malavi	Beadell et al.,2004
10	Papua New Guinea	Australasia	34	18	1.88	1.82	**1.86**	0.70	**0.01**	Malavi	Beadell et al.,2004
11	Australia	Australasia	30	15	1.00	1.56	**1.08**	0.58	**<0.001**	Malavi	Zamora-Vilchis et al.,2012
12	Russia	Europe	30	4	1.59	2.58	**1.58**	1.00	**0.04**	Malavi	Krizanauskiene et al.,2006
13	Russia	Europe	39	11	1.87	2.42	**2.04**	0.97	0.07	Malavi	Hellgren et al.,2007b
14	Sweden	Europe	18	5	0.85	1.63	**0.81**	0.60	**<0.001**	Malavi	Hellgren et al.,2007b
15	Lithuania	Europe	19	10	1.52	1.82	**1.52**	0.52	**<0.001**	Malavi	Hellgren et al.,2007b
16	Bulgaria	Europe	38	15	0.52	1.04	**0.51**	0.41	**<0.001**	Malavi	Dimitrov et al.,2010
17	Lithuania	Europe	18	6	1.39	1.74	**1.41**	0.59	**<0.001**	Malavi	Hellgren et al.,2007a
18	Portugal	Europe	7	3	0.75	1.50	**1.20**	1.64	**<0.001**	Malavi	Ventim et al.,2012
19	Israel	Middle-East	10	3	0.00	0.00	**0.00**	0.00	**<0.001**	Malavi	Martinsen et al.,2006
20	USA	North America	8	3	0.00	0.00	**0.00**	0.00	**<0.001**	Malavi	Ricklefs & Fallon 2002
21	USA	North America	12	1	3.71	2.65	**3.71**	0.87	0.57	543 F- 926R	Ricklefs 2005
22	Brazil	South America	10	1	8.58	4.91	**8.60**	1.49	1.00	343F-496R	Belo et al.,2011
23	Venezuela	South America	10	2	3.63	3.89	**3.56**	1.07	0.59	DW1-HAEMR	Belo et al.,2012
24	Brazil	South America	23	5	3.08	2.38	**3.08**	0.89	0.30	Malavi	Lacorte et al.,2013
25	Brazil	South America	11	3	3.19	2.77	**3.21**	0.74	0.34	Malavi	Lacorte et al.,2013
26	Ecuador	South America	15	7	8.66	7.41	**8.47**	2.12	1.00	3932F-3932R	Svensson-Coelho et al.,2013
27	Brazil	South America	10	2	4.11	3.43	**4.13**	0.96	0.73	3932F-3932R	Fecchio et al.,2013
28	Ecuador	South America	8	3	3.50	3.15				Malavi	This study

Discussion

The *Haemoproteus* parasite community of Wisui emerges as a very host generalist assemblage compared to those sampled in temperate areas, but shows similar levels of low specificity as those observed in other Neotropical assemblages. This result is relevant considering that the great majority of *Haemoproteus* parasites are usually more host specific. Importantly, our phylogenetic analyses support the hypothesis that different lineages have independently evolved into generalist parasites in this region. We could interpret the high amount of generalist *Haemoproteus* parasites observed in Wisui as the outcome of natural selection against host specialization, which was expected given the benefits for generalist parasites of the amplification effect, and the costs for specialists of the dilution effect in a megadiverse host environment. When host diversity is high, being a generalist would facilitate successful transmission among hosts, as parasites that thrive in a wide range of host species may benefit from higher host encounter rate (Dobson 2004; Keesing *et al.* 2006).

There is a very strong indication that Neotropical *Haemoproteus* communities show different host specificity patterns to the rest of the world, being more generalist. We found support for this conclusion in our review of parasite assemblages at different locations worldwide. For 4 of the 6 other *Haemoproteus* communities in South America we calculated an equal or higher host specificity than in Wisui reaching the highest value in the Yasuni National Park in Ecuador (Svensson-Coelho et al., 2013) and the Tocantins state in Brazil (Belo *et al.* 2011). In a review on the global diversity of avian haemosporidians Clark *et al.* (2014) showed that *Haemoproteus* diversity is higher than *Plasmodium* diversity in all geographical areas but South America. As the *Haemoproteus* communities in South America are more host generalist this could explain this observation as more ecological specialization increases biodiversity (Dyer *et al.* 2007). Intriguingly, we also found studies of tropical bird communities (such as Myanmar, Gabon or Papua New Guinea) where *Haemoproteus* parasites scored low host range values, within the typical range of temperate areas, which was a somewhat unexpected result. However, these studies sometimes covered broader geographical areas than our study in Wisui, sometimes sampling comparatively few birds per site in a large number of locations (Durrant *et al.* 2006; Ishtiaq *et al.* 2007; Beadell *et al.* 2009), which could result in the detection of more specialists which could eventually be generalists when enhancing sampling effort. The variation in host specificity for *Plasmodium* communities does not seem to show an apparent ecological or biogeographical pattern, but this requires further research.

Differences in host specificity between tropical areas could also be influenced by vector-feeding preferences (Gager *et al.* 2008; Njabo *et al.* 2011). If *Haemoproteus* vectors in South America are composed of more generalist species, this would enhance the costs of the dilution effect for specialist blood parasites, and generalist parasites could be selected for. Further research will show if vector species in the Neotropics have more generalist feeding habits than in other tropical areas which will

be essential to understand the variation of host specificity of these blood parasites. Furthermore, differences in *Haemoproteus* and *Plasmodium* vector host specificity could be the key to understand why both parasite genera show such distinct host specificity patterns.

Out of 21 parasite lineages found in Wisui, 15 had not been reported in any other host species worldwide, despite the fact that other sites have been sampled in Central and South America (Durrant et al., 2006; Merino et al., 2008; Lacorte et al., 2013). These studies, plus others in North America (where sampled birds often include Neotropical migrants), did not detect the great majority of the parasites of this study. This means that natural migrations are probably incapable to spread these pathogens outside the Neotropical area, perhaps because the periods of parasite transmission do not overlap with the presence of migrants in the area (Hellgren *et al.* 2007). The remaining *Haemoproteus* lineages found in Wisui had been detected before in birds of the Amazonian region (Schrenzel et al., 2003; Lacorte et al., 2013) , which is evidence that we may be dealing with a parasite assemblage endemic to that region and widespread at the regional level.

The fact that most parasites from Wisui are not shared with other avifaunas does not necessarily mean that this parasite assemblage has radiated locally. Neither *Haemoproteus* nor *Plasmodium* parasites found in Wisui form monophyletic groups, although a part of the diversity of the latter genus was distributed in two local radiations. *Haemoproteus* parasites include very distantly related lineages, which in most cases are closely related to parasites from the Neotropics and other regions worldwide. This result could be interpreted as a consequence of multiple colonization events involved in the assembly of this ecologically distinctive community of generalist parasites. Opportunities for colonization of new parasites can be high and parasites which can adopt a generalist strategy could be more successful colonizers in this environment than highly specialized colonizers as the latter would be ecologically filtered through the costs of a lower host encounter rate. This is especially true if their vectors are composed of generalists, which should explored in future studies.

Importantly, the *Haemoproteus* generalist lineages from Wisui share a most recent common ancestor with specialist lineages and have evolved from specialist ancestors, an observation which supports the view that a generalist strategy of host exploitation evolved repeatedly among these parasites when they faced a megadiverse host community. *Plasmodium* generalist lineages were closely related to specialist and generalist parasites from the Neotropics and other regions. Due to the more generalist nature of *Plasmodium* parasites (Valkiūnas 2005), they could also be more successful colonizers of diverse host environments through ecological fitting (Hoberg & Brooks 2008).

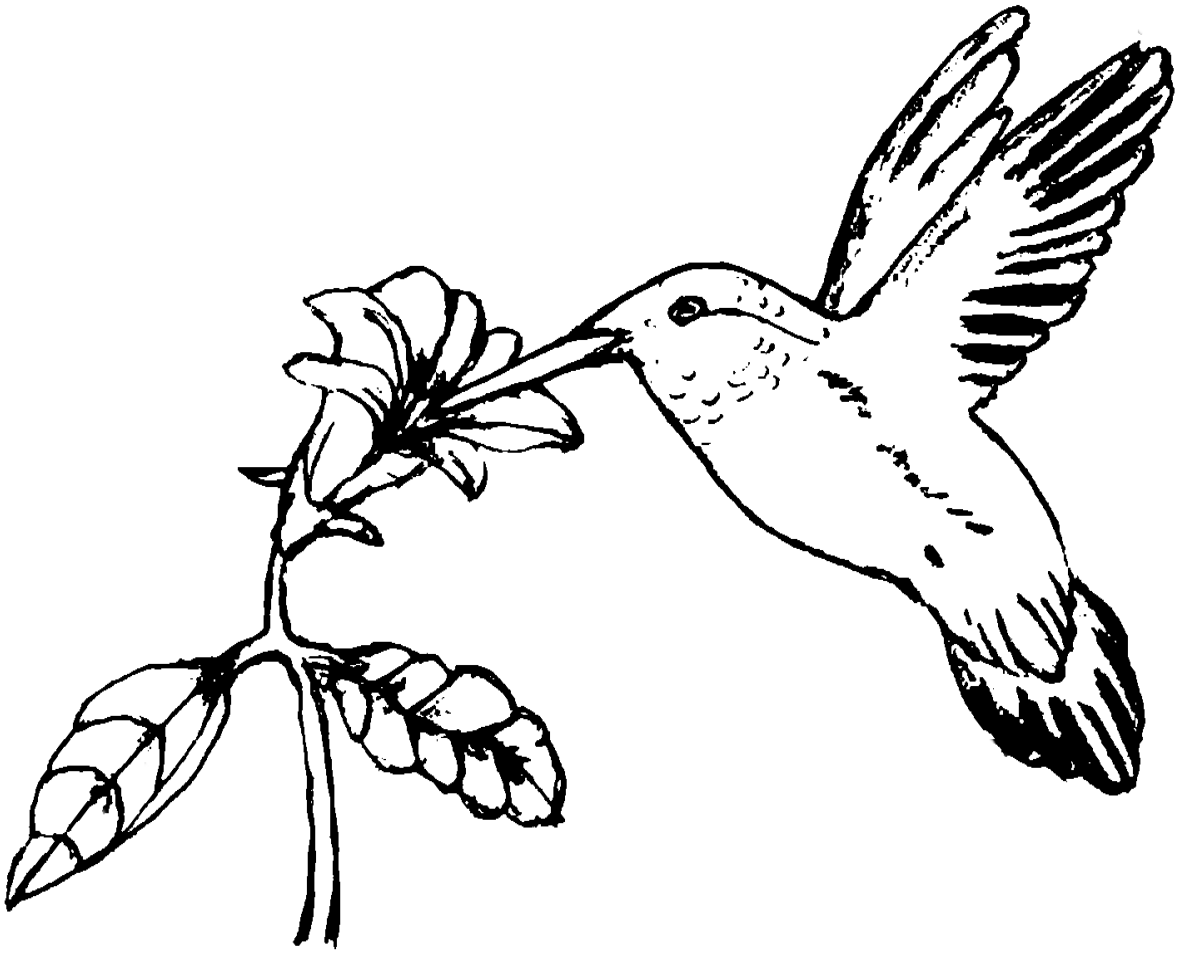
Our results support the view that a community of generalist parasites has evolved in a megadiverse host environment, an observation which may be general at least to the Andes-Amazonian region. Still, more research on the structure of parasite communities in other tropical areas in Africa and Asia, preferably conducted at a reduced spatial scale in primary habitat where bird diversity is high, is

needed to substantiate this observation as a general pattern. The Neotropics could be a reservoir of generalist blood parasites, which are best candidates to colonize new habitats where they are accidentally introduced (Ewen *et al.* 2012). Therefore, the knowledge of their global distribution and the factors influencing their local success in different host environments may greatly contribute to our understanding of the risk of disease spread and emergence.

Amazonian Barred Woodcreeper

(*Dendrocolaptes certhia*)

Chapter 2: Parasite specialization in a unique habitat: hummingbirds as reservoirs of generalist blood parasites of Andean birds.



© André Henskens

This chapter is based on the manuscript: **Moens M.A.J.**, Valkiūnas G., Paca A., Bonaccorso E., Aguirre N. & Pérez-Tris J. 2016. Parasite specialization in a unique habitat: hummingbirds as reservoirs of generalist blood parasites of Andean birds. *Journal of Animal Ecology*. In press. doi:10.1111/1365-2656.12550

Parasite specialization in a unique habitat: hummingbirds as reservoirs of generalist blood parasites of Andean birds.

Michaël A. J. Moens, Gediminas Valkiūnas, Anahi Paca, Elisa Bonaccorso, Nikolay Aguirre and Javier Pérez-Tris

Abstract

Understanding how parasites fill their ecological niches requires information on the processes involved in the colonization and exploitation of unique host species. Switching to hosts with atypical attributes may favour generalists broadening their niches, or may promote specialization and parasite diversification as the consequence.We analysed which blood parasites have successfully colonized hummingbirds, and how they have evolved to exploit such a unique habitat. We specifically asked (i) if the assemblage of *Haemoproteus* parasites of hummingbirds is the result of single or multiple colonization events, (ii) to what extent these parasites are specialized in hummingbirds or shared with other birds, and (iii) how hummingbirds contribute to sustain the populations of these parasites, in terms of both prevalence and infection intensity. We sampled 169 hummingbirds of 19 species along an elevation gradient in Southern Ecuador to analyse the host specificity, diversity and infection intensity of *Haemoproteus* by molecular and microscopy techniques. In addition 736 birds of 112 species were analysed to explore if hummingbird parasites are shared with other birds. Hummingbirds hosted a phylogenetically diverse assemblage of generalist *Haemoproteus* lineages shared with other host orders, indicating multiple colonization events. Among these parasites, *Haemoproteus witti* stood out as the most generalized. Interestingly, we found that infection intensities of this parasite were extremely low in passerines (with no detectable gametocytes) but very high in hummingbirds, with many gametocytes seen. Moreover, infection intensities of *H. witti* were positively correlated with prevalence across host species. Our results show that hummingbirds have been colonised by generalist *Haemoproteus* lineages on multiple occasions. However, one of these generalist parasites (*H. witti*) seems to be highly dependent on hummingbirds, which arise as the most relevant reservoirs in terms of both prevalence and gametocytaemia. From this perspective, this generalist parasite may be viewed as a hummingbird specialist. This challenges the current paradigm of how to measure host specialization in these parasites, which has important implications to understand disease ecology.

Keywords: Avian malaria, Ecuador, Generalist, *Haemoproteus witti*, Host specificity, Hummingbirds, Niche filling, Parasitaemia, Specialist

Introduction

How ecological niches are filled is a major question in biology (Ricklefs 2010). When vacant niches become available (for a discussion of this controversial concept see Rohde 2005), they present an opportunity for species to specialize in a new lifestyle, but can also be used by generalist species capable of exploiting the many opportunities that arise in their habitat through ecological fitting (McPeek 1996; Agosta & Klemens 2008). Whether vacant ecological niches promote the evolution of specialists or are filled by generalists may in turn determine the diversity of species and the complexity of ecological interactions that these may establish (Levine & HilleRisLambers 2009).

For parasites, host range is an important component of the ecological niche (Schmid-Hempel 2011), which is affected by various ecological and historical processes (Pérez-Tris *et al.* 2007; Ewen *et al.* 2012). Parasites may diversify with their hosts following strict co-speciation (Ricklefs *et al.* 2004), but they may also colonize new hosts when the opportunity arises. Host switching may therefore promote speciation of parasites isolated in the new host, or it may broaden the host range of parasites that thrive in different environments (Hoberg & Brooks 2008; Ricklefs *et al.* 2014a). As a consequence, parasites may differ in their degree of host specificity, ranging from specialists restricted to a single host species, to generalist capable of infecting a broad range of species (Poulin 2006; Poulin, Krasnov & Mouillot 2011). The degree of specialization of parasites will in turn determine which hosts are infected by which parasites, and therefore becomes a key factor in disease ecology.

Avian haemosporidian parasites (Haemosporida) are vector-borne protists which infect the blood and other organs of birds worldwide, which may lead to decreased fitness of their hosts (Merino *et al.* 2000; Asghar *et al.* 2015). They can be easily detected in blood samples using both light microscopy and PCR diagnostic techniques, which have revealed an extraordinary diversity of *Haemoproteus*, *Plasmodium*, and *Leucocytozoon* parasite lineages worldwide (Valkiūnas 2005; Bensch *et al.* 2009). Host specificity of avian malaria parasites differs greatly both among and within parasite genera (Hellgren *et al.* 2009). Although many studies have improved our understanding of this variation (Gager *et al.* 2008; Hellgren *et al.* 2009; Medeiros, Ellis & Ricklefs 2014; Ricklefs *et al.* 2014a) the ecological and evolutionary determinants of host breadth strategies are still poorly understood. For example, there is evidence that avian malaria parasites have diversified mainly by host switching and co-speciation of highly specialist parasites (Ricklefs *et al.* 2014a), but there are cases of diversification within single host species (Ricklefs *et al.* 2005; Pérez-Tris *et al.* 2007), and generalist parasites have evolved several times in the group (Moens & Pérez-Tris 2016). However, we still do not know much about the processes involved in the colonization of hosts that provide unique niches to parasites. These may vary in their degree of competence as hosts, thereby affecting the network of

host-parasite interactions and the ecology of disease transmission at the community level (Ostfeld & Keesing 2012).

The explosive radiation of hummingbirds provides an ideal system to investigate the evolution of host specificity among avian Haemosporidia. Hummingbirds are a special habitat for these parasites given their high metabolic rates, energy-demanding flight, and small body size. Hummingbirds possess special adaptations such as high relative heart and lung volumes, mitochondrial respiration rates, and capillary volume densities, which makes them distinctive in the vertebrate world (Suarez *et al.* 1991). Hummingbirds also have small erythrocytes capable of flowing through numerous narrow capillaries and with a high surface to volume ratio, which guarantees the quick exchange of respiratory gases (Opazo *et al.* 2005). Moreover, their blood has the highest erythrocyte level among birds, reaching values that may exceed 6.5 million red blood cells per microliter (Glomski & Pica 2011).

All the above attributes make hummingbirds special environments for avian blood parasites, yet we still do not know the properties of parasite assemblages that have colonised this unique habitat. The outcome of that process may have depended on the capability of parasites to adapt to hummingbirds. On the one hand, severe anaemia caused by parasites could be problematic for birds whose lifestyle depends on high levels of oxygenation. This constraint may have driven the evolution of a radiation of hummingbird specialist parasites, which minimise their infection intensities in order to survive in such delicate hosts. Alternatively, parasite specialization may have been prevented if hummingbirds die soon and are poor reservoirs as a consequence (Bull & Lauring 2014). In that case, the parasites found in hummingbirds could be generalists that regularly spillover from other sympatric bird species.

We studied the diversity, host specificity, prevalence and infection intensities of *Haemoproteus* (Haemosporidia, Haemoprotidae) parasites from different avian communities along an elevational gradient in the South Ecuadorian Andes, an area known for its high hummingbird diversity (McGuire *et al.* 2014). Despite the fact that the Neotropics hold more than a third of the world bird species (Myers *et al.* 2000), only 29 out of 140 morphologically distinct species of *Haemoproteus* have been found in this region (Valkiūnas 2005). To date, only three species of *Haemoproteus* parasites have been described in hummingbirds: *H. witti*, *H. archilochus*, and *H. trochili* found in different species (White, Bennett & Williams 1979; Valkiūnas 2005; González *et al.* 2015). These species were described based on unique morphological characters by microscopy, but their evolutionary relationships remain unknown. Here, we will analyse the diversity of *Haemoproteus* parasites using DNA barcoding and microscopy screening, two techniques which combined provide much insight in avian blood parasite studies (Bensch *et al.* 2009).

Our objectives were twofold. First we analysed the evolutionary process that shaped the diversity and host specificity of *Haemoproteus* parasites in Andean hummingbirds. Hummingbird parasites could have diversified through host-parasite co-speciation or host switching (Ricklefs *et al.* 2004, 2014a)

after one or various colonization events, in which case they are expected to form one or various clades within the diversity of *Haemoproteus* (Ricklefs *et al.* 2005; Pérez-Tris *et al.* 2007). Alternatively, the parasites may have colonized hummingbirds on different, more recent occasions in evolutionary history, in which case they will have diverse phylogenetic ancestry. Within hummingbirds, parasites may have become specialists (infecting a single host species each), or they may have remained infecting different bird species (a generalist strategy which may include other bird families). Second, we analysed the ecological role of hummingbirds as parasite reservoirs. These unique hosts will be important for any specialist parasite, but they may play different roles as reservoirs of the generalist parasites they may share with other birds. We used parasite prevalence to score the contribution of hummingbirds and other birds to sustain the population of infected hosts available to vectors. To further examine the role of different bird species as parasite reservoirs, we focused on *H. witti*, a common parasite of hummingbirds which was the most generalist and abundant parasite in our study (see Results). We analysed the relationships between prevalence and intensity of infection, a measure of the ability of this parasite to multiply within hosts. A positive correlation would support the view that birds with high prevalence are especially important for the parasite, because they contribute many reservoirs with many parasites available to blood-sucking insects, which may increase transmission to vectors (Cornet *et al.* 2014; Pigeault *et al.* 2015). However, a negative correlation between prevalence and intensity of infection would support alternative scenarios in which rare transmission events are associated with high parasite virulence, or infected hosts are rapidly purged by mortality (Poulin 2006). By scoring the importance of hummingbirds as reservoirs of the most common parasite in this community, our study will shed light on the role of niche diversity on the evolutionary ecology of avian blood parasites.

Materials and methods

Study area and field methods

The study was conducted along an elevation gradient in the Podocarpus National Park in South Ecuador, at four different altitudes (1500, 2000, 2500, and 3000 meters above sea level.) during 6 consecutive months (June–November 2012), visiting all sites four times with a 30-day interval. Birds were captured using 15 mist nets (12 m long × 2.5 m high, 25 mm mesh) and species were determined according to Ridgely & Greenfield (2006), and following the updated nomenclature proposed by the South American classification committee. We took standard body measurements of all captured birds (wing, tail and tarsus length, and weight), and photographed individuals to confirm difficult species identifications. We collected blood samples from all birds (5-80 µl, depending on body size) by puncture of the brachial, jugular, or metatarsal vein. We used part of the blood to make two blood smears, which were air dried and fixed in absolute ethanol. The remaining blood was kept in absolute ethanol to preserve DNA, at ambient temperature in the field and at -20 ºC until molecular analysis.

Once processed, birds were marked by feather tip cuts or rings to control recapture data, and released unharmed at the site of capture. Bird sampling was performed in compliance with Ecuadorian and European regulations and with the authorizations issued by the Ecuadorian Ministry of Environment of the Loja province under research permit number 009-2012-IC-FAU-DPL-MA.

Laboratory methods

Blood smears were stained with Giemsa solution (pH 7.2) for one hour. Each blood smear was examined with a light microscope (LEICA DM2500). We first scanned smears at 400× to search for different types of intracellular and extracellular blood parasites. Then we screened them at 1000× focusing on intra-erythrocytic parasites, until 200 fields were inspected which corresponds to approximately 100,000 erythrocytes. We determined intensity of parasitaemia by counting infected erythrocytes over a total of 10,000 red blood cells. We identified the parasites to morphospecies according to White et al. (1979) and Valkiūnas (2005). The blood films were compared with the type material of *H. witti* and *H. trochilis* deposited in the Collection of the International Reference Centre for Avian Haematozoa (IRCAH) at the Queensland Museum, Queensland, Australia.

We extracted total DNA from blood samples with a standard ammonium acetate protocol. For each parasite, the MalAvi barcode for avian haemosporidians was amplified (479 bp of the mitochondrial cytochrome *b* gene; (Bensch *et al.* 2009)), which has been sequenced for the majority of the known diversity of these parasites. DNA quality was verified by amplifying bird sexing markers for every bird individual (Fridolfsson & Ellegren 1999). We screened for parasite infections using the nested PCR protocol (Waldenström *et al.* 2004), which was specifically designed to amplify *Haemoproteus* and *Plasmodium* DNA. The first PCR, in a total volume of 25 µl, included 25 ng of total genomic DNA, 1.25 mM of each dNTP, 1.5 mM MgCl2, 10 µM of the primers HaemNF and HaemNR2 and 0.5 units AmpliTaq DNA polymerase (Applied Biosystems). The following thermal profile was used: a denaturation step of 94 °C for 3 min, 20 amplification cycles of 30 s at 94 °C, 30 s at 50 °C and 45 s at 72 °C, and a final extension step for 10 min at 72 °C. We used 1 µl of the PCR product as the template for the second PCR, with primers HaemF and HaemR2 and the same reaction conditions, except for using 35 amplification cycles (Waldenström *et al.* 2004). We visualized 4 µl of the final PCR product on a 2% agarose gel stained with GELRED. A negative control was included in each row of the PCR plate, which also included a positive control. Negative controls (n = 143) never produced positive results. PCR products were precipitated by adding 11 µl of 8M NH4Ac and 33 µl absolute ethanol, and diluted in 15–20 µl of water. We sequenced them from both ends (using primers HaemF and HaemR2) with a dye-terminator AmpliCycle sequencing kit and an ABI PRISM^{TM} 3700 sequencing robot (Applied Biosystems, UK).

We quantified parasitaemia intensities of *H. witti* of both visible and submicroscopic parasite infections using real-time quantitative PCR (qPCR) in a 7900HT Fast Real-Time PCR System

(Applied Biosystems). We diluted extracted DNA to 1 ng/µl and quantified infection intensities by using general primers 343F and 496R, which amplify a 154 nucleotide segment of RNA-coding mitochondrial DNA of the parasite (Fallon *et al.* 2003). For quantifying total DNA contents, we used the host-specific primers sfsr3Fb and sfsr3Rb to amplify an ultra-conserved single-copy nuclear sequence (Asghar *et al.* 2015). Each reaction of 10 µl included 2.5 µl DNA template (1 ng/µl), 2.5 µl primers at 1.2 µM and 5 µl of Master Mix (FastStart Universal SYBR Green Master (Rox); Roche Diagnostics). Thermal cycles started with an initial incubation at 95 ºC for 10 min followed by 40 cycles at 95 ºC for 15 s and 57 ºC for 1 min. Each DNA sample was run in duplicate and the average value was used for further analysis. Standard curves were produced by diluting samples in four step - 10× dilutions (2.5 ng to 0.0025 ng of DNA per well). Standard curves were around 95% qPCR efficiency.

We evaluated if parasitaemia intensities measured by microscopy and qPCR are consistent by a linear regression analysis in R. We analysed the relationship between prevalence and intensity of infection among species using qPCR data to measure parasitaemia. To reduce the impact of influential values upon our estimates of prevalence and intensity of infection (a problem associated with low per-species sample sizes), we computed average values from 1000 databases obtained by bootstrap (with replacement) of the original data, and discarded species with less than five sampled host individuals (thereby avoiding very unreliable prevalence estimates due to low sample size). These data were analysed using beta regression models as implemented in the R package "betareg" (Cribari-Neto & Zeileis 2010) ,assuming the dependent variable (prevalence, which takes values in the interval [0, 1]) to be beta-distributed, and using a logit link function.

Phylogenetic analyses

DNA sequences were manually aligned and edited using BioEdit (Hall 1999). Sequences differing by one nucleotide substitution were considered to represent unique lineages (Bensch *et al.* 2004, 2009). We identified parasite lineages by means of local BLAST analyses of the MalAvi database (http://mbio-serv2.mbioekol.lu.se/Malavi/; Bensch et al. 2009) and GenBank (http://www.ncbi.nlm.nih.gov/genbank/). We reconstructed the phylogenetic relationships of the lineages found in hummingbirds placing them in the tree that included all morphologically characterised *Haemoproteus* parasites with complete MalAvi barcodes (479 bp amplified with primers HaemF and HaemR2). We applied a Bayesian analysis with BEAST 2.0 software (Bouckaert *et al.* 2014), using the most appropriate substitution model according to the Bayesian Information Criterion implemented in PartitionFinder (Lanfear *et al.* 2014): HKY+I+G. We specified the parameters for the BEAST-run in BEAUTI 2.0 (Bouckaert *et al.* 2014) and MCMCs were run for 10^9 generations, sampling every 100,000 trees. A Yule speciation prior and strict clock model were used as our data could not reject this model based on the histogram of ucdl.stdev values in Tracer 1.5

(http://tree.bio.ed.ac.uk/software/tracer/); this choice was further supported by the fact that a molecular clock has recently been estimated for malaria parasites (Ricklefs & Outlaw 2010). Estimated sample sizes were all higher than 200. The 10,000 resulting trees were summarized with TreeAnnotator v2.1.2 (http://beast.bio.ed.ac.uk/treeannotator) and the phylogenies with the posterior probabilities of the nodes were displayed in FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) for further analysis.

We calculated a host specificity index for every parasite in the phylogeny, which we further refer to as the S^*_{TD} index (Hellgren *et al.* 2009). This index measures a host range for each parasite considering the diversity of host species, the taxonomic distance between host species and its variance (Hellgren *et al.* 2009). It was calculated as follows:

$$S^*_{TD} = S_{TD} + \frac{s-1}{1 + Var{S_{TD}}^{s}}$$

$$S_{TD} = 2\frac{\sum\sum_{i<j}\omega_{ij}}{s(s-1)}$$

$$VarS_{TD} = \frac{\sum\sum_{i\neq j}\left(\omega_{ij} - S_{TD}\right)^2}{s(s-1)}$$

where ωij is the taxonomic distance between host species i and j (i.e., how many taxonomical steps need to be taken to get to their most recent common ancestor) and *s* is the number of host species infected by the parasite (Hellgren *et al.* 2009).

We define generalist parasites as those found in two or more host species, as opposed to specialists found only in one host species each. The higher the S^*_{TD} value the more generalist a parasite is considered. We compared the relative host specificity of every parasite in this study by calculating a S^*_{TD} value for every *Haemoproteus* lineage of the MalAvi database (last accessed April 2015). Finally, in order to better illustrate the phylogenetic host range of *H. witti*, we constructed a phylogeny of all sampled bird species through BIRDTREE (Jetz *et al.* 2012) and plotted the occurrence, prevalence and intensity of *H. witti* on all infected bird species. The phylogenetic tree was generated by request of a subset of the whole tree distribution from the "Ericson All Species" source and by selecting all bird species which were sampled in this study. We requested 100 trees and a consensus tree was built for further analysis. A detailed summary of the methods used to generate the original phylogeny can be found in Jetz *et al.* (2012).

Results

Parasite prevalence, diversity and host specificity

We screened 169 hummingbirds from 19 different species by PCR and light microscopy (Table 1; Table 2, Appendix S1). Additionally, 736 birds of 112 passerine and non-passerine species were examined by both techniques (Appendix S1). Using PCR, we retrieved four distinct *Haemoproteus* lineages from 50 hummingbirds belonging to 11 species (Table 1, Table 2). All four lineages classified as generalists according to their calculated S^*_{TD} value (Fig.1) and have been already detected in other birds in South America (Lacorte *et al.* 2013; Galen & Witt 2014; González *et al.* 2015). Only one of these four lineages (TROAED20 in the MalAvi database; (Bensch *et al.* 2009; Galen & Witt 2014) was detected by light microscopy in hummingbirds, as shown by observation of circulating gametocytes. This particular lineage was detected by PCR in 90 individuals belonging to 10 hummingbird species and 26 passerine species (Table 1, Fig. 2), scoring S^*_{TD} = 43.3 according to its observed host-range (Fig. 1 and 2). The prevalence of this lineage based on PCR data was high in hummingbird species, ranging from 14.3% in *Metallura tyrianthina* to 80% in *Boissoneaua matthewsii* (Table 1, Fig. 2). Interestingly, after blood slide screening of all 90 PCR positive individuals of this lineage, gametocytes were only detected in 30 hummingbirds of 7 species and were never detected in passerine species despite exhaustive examination (Table 1, Fig. 2).

We identified the TROAED20 lineage as *H. witti* (White *et al.* 1979), after comparing our slides with the type material. In all seven hummingbird species with parasites detected by microscopy, the gametocytes showed similar morphology in the position of the parasite nucleus, the number of pigment granules, the displacement of host cell nucleus and the growing pattern of young, medium-grown, and mature gametocytes (Fig. S1). Based on comparison of the type specimens and our material, we added new characteristics of the young gametocytes to the original description (Appendix S2). As the type material of *H. witti* (accession nr. 62876) is fading and pigment granules are poorly visible in this slide (Appendix S2), we designated new voucher material from five different hummingbird species (G466184-466188). The vouchers will be deposited in the IRCAH collection of the Queensland Museum. The three other *Haemoproteus* lineages were detected by PCR in different species of hummingbirds and passerines but gametocytes of these parasites were seen only in passerines (Table 2).

Table 1: List of host species where *Haemoproteus witti* was detected. Infection intensity measured by qPCR and microscopy are presented for every host species (mean ± SD). Hummingbird species are highlighted in bold.

Host Order and Species	***No. Ind. Captured***	***No. PCR Positives***	***No. Microscopy Positives***	***Prevalence PCR***	***Prevalence Microscopy***	***qPCR Parasitaemia Mean±SD***	***Microscopy Parasitaemia Mean±SD***	***Bootstrap Prevalence Mean±SE***	***Bootstrap Parasitaemia Mean±SE***
Apodiformes									
Adelomyia melanogenys	31	8	5	25.8	16.3	0.082 ± 0.076	1.538 ± 1.15	0.256 ± 0.002	4.273 ± 0.036
Amazilia alticola	17	3	0	17.6	0	0.016 ± 0.022	0	0.18 ± 0.003	2.117 ± 0.049
Boissoneaua matthewsii	5	4	4	80	80	1.30 ± 0.41	8.762 ± 2.95	0.807 ± 0.006	9.428 ± 0.005
Coeligena iris	27	19	16	70.4	59.3	0.404 ± 0.519	5.716 ± 7.14	0.703 ± 0.003	7.191 ± 0.014
Coeligena torquata	8	1	0	12.5	0	/	0	0.125 ± 0.004	
Colibri thalassinus	4	1	1	25	25	0.457	5.3	0.245 ± 0.007	8.429
Heliangelus viola	13	3	2	23.1	15.4	0.034 ± 0.039	0.63 ± 0.76	0.232 ± 0.004	5.384 ± 0.017
Lafresnaya lafresnayi	6	4	1	66.7	16.7	0.06 ± 0.102	5.5	0.661 ± 0.006	3.132 ± 0.042
Metallura tyrianthina	7	1	1	14.3	14.3	0.061	0.25	0.141 ± 0.004	6.411
Phaethornis griseoregularis	11	2	0	18.2	0	$4 \times 10^{-5} \pm 3 \times 10^{-5}$	0	0.182 ± 0.004	0.222 ± 0.005
Passeriformes									
Amblycercus holosericeus	3	1	0	33.3	0	0.017	0	0.327 ± 0.009	5.167
Arremon torquatus	14	2	0	14.3	0	0.011 ± 0.015	0	0.147 ± 0.003	3.585 ± 0.041
Basileuterus trifasciatus	16	2	0	12.5	0	0.048 ± 0.066	0	0.123 ± 0.003	5.019 ± 0.042
Campylorhynchus fasciatus	3	1	0	33.3	0	1.4×10^{-4}	0	0.333 ± 0.008	0.861
Cinnycerthia unirufa	15	1	0	6.7	0	3.1×10^{-4}	0	0.067 ± 0.002	1.406
Cranioleuca antisiensis	2	1	0	50	0	/	0	0.515 ± 0.011	/
Diglossa caerulescens	4	1	0	25	0	0.002	0	0.247 ± 0.007	2.926
Elaenia albiceps	30	1	0	3.3	0	/	0	0.035 ± 0.001	/
Elaenia pallatangae	3	1	0	33.3	0	/	0	0.321 ± 0.008	/
Furnarius leucopus	9	2	0	22.2	0	$8 \times 10^{-4} \pm 5 \times 10^{-5}$	0	0.224 ± 0.005	2.195 ± 0.001
Hellmayrea gularis	9	1	0	11.1	0	0.067	0	0.111 ± 0.003	6.519
Icterus mesomelas	1	1	0	100	0	4×10^{-5}	0	1 ± 0	0.363
Lepidocolaptes lacrymiger	9	1	0	11.1	0	1.2×10^{-4}	0	0.116 ± 0.003	0.802

Mionectes striaticollis	81	5	0	6.2	0	$9 \times 10^{-5} \pm 8 \times 10^{-5}$	0	0.061 ± 0.001	0.512 ± 0.006
Myadestes ralloides	54	6	0	11.1	0	$2 \times 10^{-5} \pm 3 \times 10^{-5}$	0	0.109 ± 0.001	0.128 ± 0.003
Myioborus miniatus	9	1	0	11.1	0	/	0	0.112 ± 0.003	/
Myiothlypis coronata	41	6	0	14.6	0	$7 \times 10^{-4} \pm 0.001$	0	0.142 ± 0.002	0.525 ± 0.013
Myiothlypis fraseri	13	1	0	7.7	0	1.3×10^{-4}	0	0.078 ± 0.002	0.854
Myiothlypis nigrocristatus	14	2	0	14.3	0	$2 \times 10^{-4} \pm 2 \times 10^{-4}$	0	0.146 ± 0.003	0.903 ± 0.011
Ochthoeca rufipectoralis	9	1	0	11.1	0	/	0	0.114 ± 0.003	/
Pipreola riefferii	14	1	0	7.1	0	0.003	0	0.069 ± 0.002	3.42
Synallaxis azarae	30	1	0	3.3	0	7×10^{-5}	0	0.035 ± 0.001	0.525
Thraupis cyanocephala	5	1	0	20	0	0.002	0	0.198 ± 0.005	2.786
Tiaris obscurus	19	1	0	5.3	0	1.2×10^{-4}	0	0.051 ± 0.002	0.782
Turdus nigriceps	12	1	0	8.3	0	7×10^{-4}	0	0.076 ± 0.002	2.075
Turdus serranus	11	1	0	9.1	0	4×10^{-4}	0	0.093 ± 0.003	1.636
Total: 36 species	**559**	**90**	**30**						

Table 2: List of host species in which *Haemoproteus* lineages PAPOL01, TANIG01, TROAED15 were detected by PCR and light microscopy. Hummingbird species are highlighted in bold.

Host order and species	Lineage	Nr. individuals captured	PCR positives	Nr. individuals with gametocytes	Prevalence PCR	Prevalence Microscopy
Apodiformes						
Adelomyia melanogenys	TROAED15	31	1	0	3.2	0
Coeligena iris	PAPOL01	27	1	0	3.7	0
Coeligena torquata	TROAED15	8	1	0	12.5	0
Eriocnemis vestita	TANIG01	21	1	0	4.8	0
Passeriformes						
Basileuterus trifasciatus	PAPOL01	16	1	0	6.3	0
Diglossa albilatera	TROAED15	25	4	4	16	14.8
Diglossa cyanea	TROAED15	20	7	7	35	33.3
Myiothlypis fraseri	PAPOL01	13	1	0	7.7	0
Pachyramphus albogriseus	PAPOL01	3	2	1	66.7	33
Tangara vassorii	TANIG01	2	2	2	100	100
Tyrannus melancholicus	PAPOL01	4	1	0	25	0

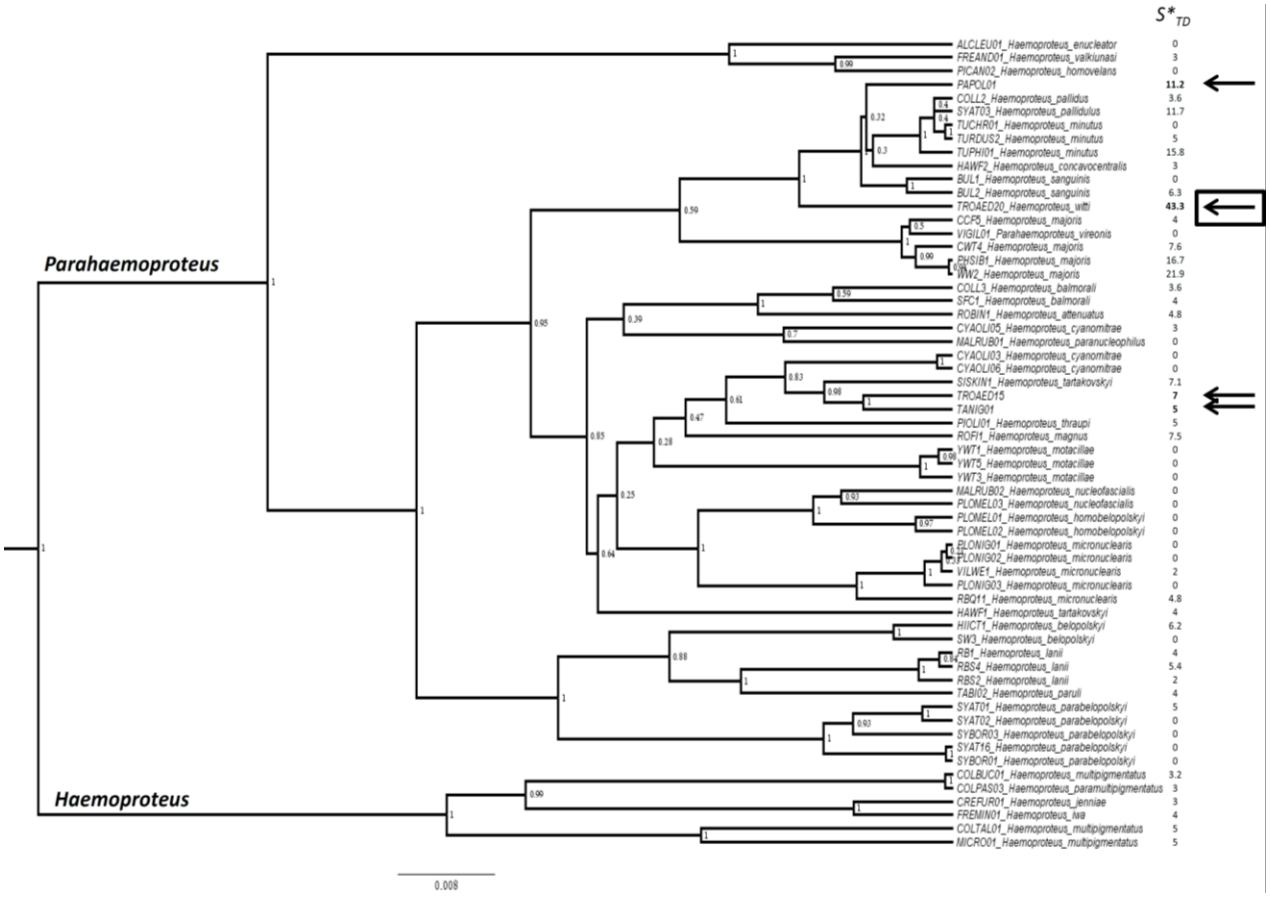


Figure 1: Bayesian phylogeny of *Haemoproteus* species with available cyt. *b* sequences and the four lineages found in this study. Posterior probabilities of branch support are shown. The *Parahaemoproteus* and *Haemoproteus* clades are indicated by their names, and S^*_{TD} values are shown next to the species. The arrows indicate *Haemoproteus* lineages found in hummingbirds in this study. *Haemoproteus witti* is marked with the arrow in the box.

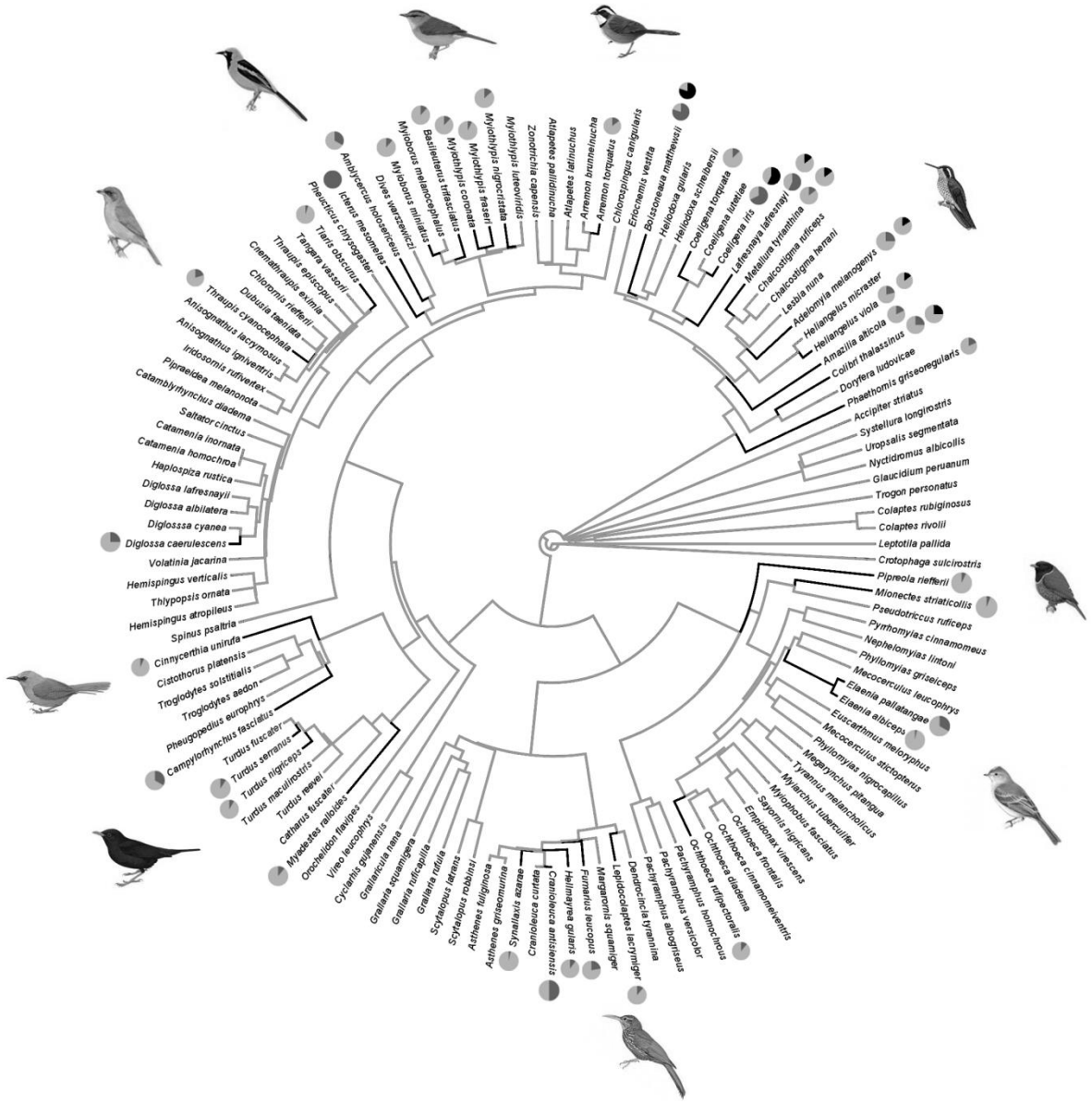


Figure 2: Avian phylogeny of the sampled bird species, showing the ones which were positive for *Haemoproteus witti* (black branches). Circular diagrams represent infection prevalence both by PCR (grey) and microscopy (black). One species representative of each bird family positive to *H. witti* is shown with an illustration.

Phylogenetic relationships

The resulting phylogeny shows that *H. witti* (TROAED20) and the other three hummingbird lineages belong to the *Parahaemoproteus* clade (Fig.1). TROAED20 and PAPOL01 form part of a clade with very generalist *Haemoproteus* parasites (Fig. 1). TANIG01 and TROAED15 are closely related to *H. tartakovskyi* and also belong to a more generalist clade (Fig. 1). TROAED20 has also been detected in Peru in 40 different hummingbird species throughout several localities in the Andes and Amazon (Genbank PopSet: 475644377). Three hummingbird species were positive for this lineage in Colombia (González *et al.* 2015) and one in Ecuador (Harrigan *et al.* 2014). PAPOL01 was detected

in Brazil in *Pachyramphus polychopterus* (Lacorte *et al.* 2013). Lineage TROAED15 was found in Peru in *Troglodytes aedon* (Galen & Witt 2014) and TANIG01 was detected in various tanager species in Colombia (González *et al.* 2015). The new lineage data were uploaded to the MalAvi database, and sequences were deposited in GenBank with accession numbers KU364540-86.

Parasite infection intensities

We performed qPCR for 87 individuals which were PCR positive for *H. witti*. The intensity of infection measured by light microscopy was positively correlated with the intensities detected by qPCR (both variables log-transformed: $R = 0.89$; $n = 87$; $p < 0.001$; Fig. 3A). The correlation improved when we removed 24 birds which were also positive for *Leucocytozoon spp.* (none was co-infected with *Plasmodium*), which could interfere with qPCR results ($R = 0.91$; $n = 63$; $p < 0.001$; Fig. 3B). The result remained when we removed 18 PCR positive infections that were under the detection threshold of qPCR in two trials (6 from hummingbirds and 12 from other species, one of which was positive for microscopy with very low intensity; $R = 0.90$; $n = 45$; $p < 0.001$). In order to rule out possible contamination in these 18 samples a normal nested PCR was repeated for 13 of 18 infections independently with positive and negative controls and all tested positive again. Note that we tested 143 other negative control samples, which gives a rate of false positives of $p < 0.01$. Finally, we found a positive correlation of the mean qPCR intensity of infected birds and PCR prevalence across host species (intensities log-transformed, beta regression estimate = 0.24; $z = 4.44$; $p < 0.0001$; Fig. 3C). This positive correlation between infection intensity and prevalence across host species was to some extent influenced by a clear difference between hummingbirds and other species, both in prevalence (bootstrapped values: 36.5 ± 0.05 on average, 247% higher than in other bird species) and in intensity of infection of *H. witti* (bootstrapped values, log-transformed: 4.24 ± 0.77 on average, 139% higher than in other bird species; Fig. 3). However, when host order (hummingbird vs. passerines) was included as a factor in the beta regression model, intensity of infection explained more variation in prevalence than type of host (qPCR intensity: estimate = 0.18; $z = 3.32$; $p < 0.001$; type of species: $z = 3.15$; $p = 0.002$). The interaction between infection intensity and host type was not significant (estimate = 0.16; $z = 1.41$; $p = 0.16$), indicating that the relationship between intensity of infection and prevalence of *H. witti* had a common slope in hummingbirds and other hosts (Fig. 3C).

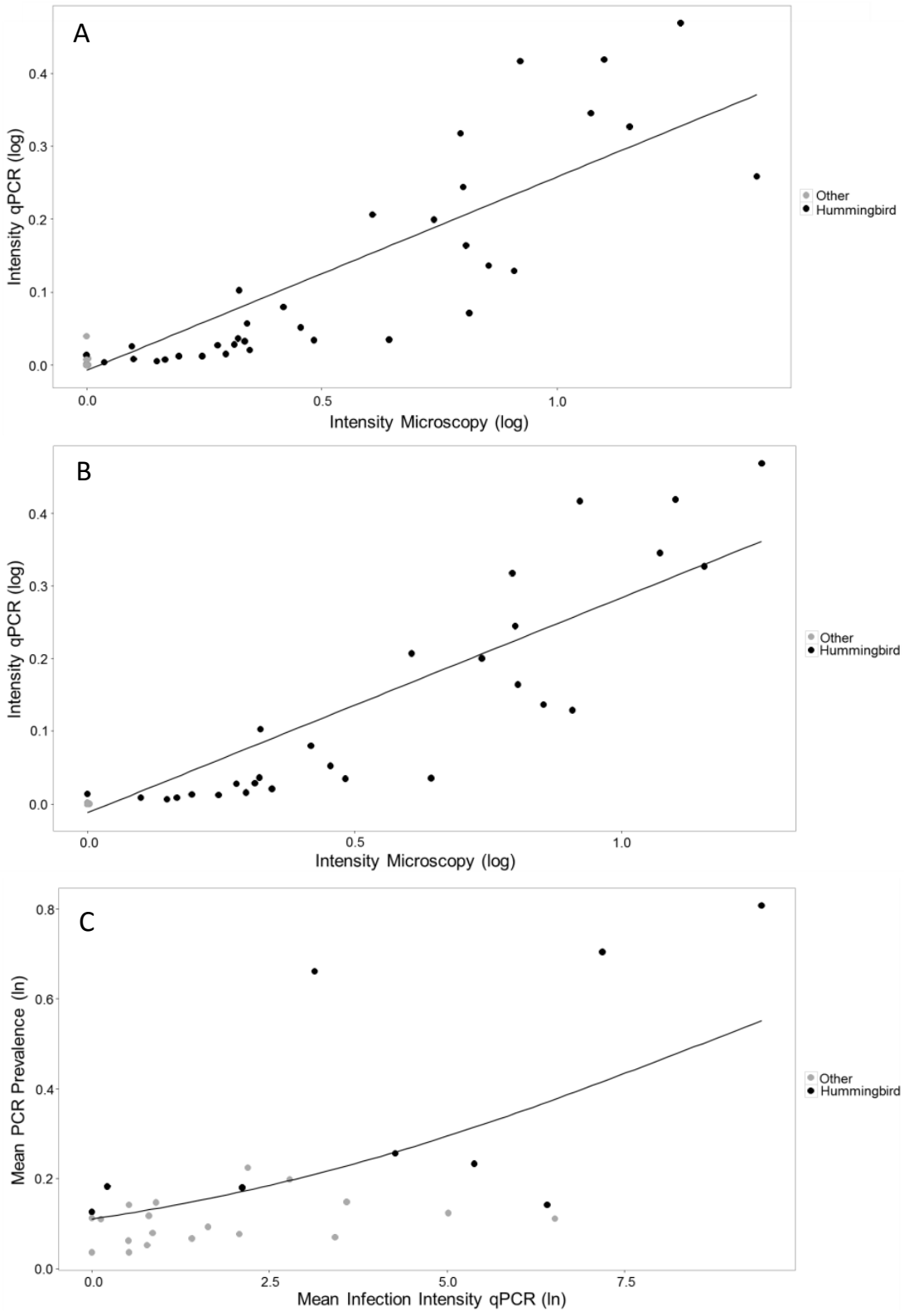


Figure 3: Relationships between the parasitaemia of *Haemoproteus witti* measured by microscopy and qPCR. In (A) 87 individuals are included in the analysis while in (B) all individuals co-infected with *Leucocytozoon* were excluded. A beta regression also shows the correlation between mean parasitaemia intensity (as determined by qPCR, bootstrap means) and prevalence (as derived from PCR data, bootstrap means), across all bird species with more than five individuals sampled (C). Hummingbirds are represented by black dots while other species are depicted as grey dots.

Discussion

Our study produced three key results to understand the ecology and evolution of blood parasite assemblages of hummingbirds. First, we show that hummingbirds host a low diversity of *Haemoproteus* parasites with diverse phylogenetic ancestry in the Ecuadorian Andes. This result supports the hypothesis that host-switching has been an important process in the evolution of the parasite assemblage of this unique family of birds. Second, all *Haemoproteus* parasite lineages found in hummingbirds are generalists shared with birds of other families. This observation supports the idea that ecological fitting rather than specialization has governed the filling of the unique hummingbird niche. Third, despite being a generalist parasite, the most abundant parasite species in this community (*H. witti*) reaches its highest prevalence and intensity of infection in hummingbirds, which suggests they are the most important reservoirs of this parasite at the community level. These results help us to understand the evolution of a unique community of avian blood parasites, composed of generalist parasites with different dependence on hummingbirds as main hosts.

Other studies have shown that host switching is a major mechanism in the diversification of avian blood parasites (Ricklefs et al. 2004, 2014). Host-switching is promoted when parasites are transmitted by vectors with generalist feeding habits (Gager *et al.* 2008; Medeiros, Hamer & Ricklefs 2013). According to this, the host generalist parasites found in hummingbirds are likely to also be vector generalists, an idea that warrants further research. Nevertheless, the absence of strict isolation in different host species promotes gene flow between parasite populations, limiting speciation as the consequence (Ricklefs *et al.* 2014a).

The fact that all parasite lineages found in hummingbirds were observed in other host orders suggests that hummingbirds do not promote parasite specialization, but are affected by parasites that may spillover from other sympatric species. These opportunistic parasites may have better chances to successfully invade poorly defended hosts (Møller, Christe & Garamszegi 2005). Hummingbirds could be poorly protected if the high energetic cost of their lifestyle compromises immune function (Sheldon & Verhulst 1996; Schmid-Hempel 2011). This could explain their apparent propensity to host phylogenetically distantly related generalist parasites with little relevance as hosts. Thus, three out of four parasites found (PAPOL01, TANIG01, TROAED15) had low prevalence and intensity of infection in hummingbirds compared to other bird species: in all instances they represented single cases not detected by microscopy. Whether these cases represented too light infections to be detected by microscopy or abortive infections of atypical hosts (Olias et al. 2011), the conclusion that hummingbirds are poor reservoirs for these parasites remains unchanged.

However, parasite spillover cannot explain the biological characteristics of *H. witti*. In the bird community investigated here and in other Neotropical areas alike, this parasite takes the generalist strategy of host exploitation to an extreme. In our study area, *H. witti* infects 36 species of 11 families.

A positive relationship between prevalence and intensity of infection across species suggests that the most heavily infected bird species are the most important reservoirs for this parasite, which is more capable of colonizing and multiply successfully within hummingbirds than in other birds. Prevalence represents the contribution of each species to the population of infected hosts, and transmission to vectors may be favoured by high gametocytaemia, as shown in experimental studies of avian malaria parasites (Cornet et al. 2014). Although the small sample size available per species compels us to take our data of prevalence and intensity with appropriate caution, it is important to notice that hummingbirds consistently show the highest prevalence and infection intensities of *H. witti*, according to both PCR and microscopy data. This result also rules out the possibility that the relationship between prevalence and intensity was driven by low probability of detection of very light infections. In fact, we only detected gametocytes of *H. witti* in hummingbirds, failing to see them in 44 PCR positive infections of 26 passerine species. This observation suggests that hummingbirds may be particularly important reservoirs of this parasite. In fact, to date gametocytes of *H. witti* were known from hummingbirds alone (Valkiūnas 2005; González *et al.* 2015). However, our PCR results placed this *Haemoproteus* lineage as one of the few *Parahaemoproteus* that occur across orders, and the most host generalized in the MalAvi database (Bensch *et al.* 2009).

Many studies on the specificity of avian malaria parasites consider the range of host species in which a parasite is found as the basis to compute its specificity. From this perspective, *H. witti* would be viewed as a super-generalist parasite. However, in light of prevalence and parasitaemia, it can be viewed as a parasite that has adapted to exploit hummingbirds. We found additional evidence in support of this idea by comparing infection status of birds recaptured throughout the year. In 10 passerines recaptured, *H. witti* only occurred once in every individual over one to three months, suggesting the parasite appears in the blood and disappears below detection threshold over this period. In contrast, three recaptured hummingbirds maintained infection over the same time period. This difference was statistically significant (Fisher exact test: $p = 0.014$), and further supports the view that hummingbirds play a more relevant role than other birds as reservoirs of *H. witti*.

The observation of a large proportion of hummingbirds facing high parasitaemia was somewhat unexpected, and how these energetically compromised birds manage to afford such intense parasite exploitation remains an open question. As a possible explanation, hummingbirds might rely on tolerance rather than resistance mechanisms to maintain fitness under intense parasite exploitation, while other host species could keep *H. witti* at low levels by mounting more costly immune responses (Bonneaud *et al.* 2003). If this results in many hummingbirds being around as reservoir hosts with high parasite load, *H. witti* could be quite successful locally without very sophisticated means of evading a large diversity of host immune systems. Non-hummingbird hosts could keep the parasite at low levels, eliminate it or die from infection without compromising transmission of the parasite. If

this possibility proves true, we would be describing a new mechanism for parasite specialization in a unique ecological niche.

It is surprising we did not find one single passerine with circulating gametocytes of *H. witti*. As we have screened approximately 100,000 erythrocytes in all birds, the possibility of not detecting circulating gametocytes is still acceptable, as PCR can detect infections with less than one gametocyte in one million host cells (Hellgren *et al.* 2004). Importantly, our qPCR results from *H. witti* reveal the presence of very small amounts of parasite DNA in the blood of passerine birds, which is compatible with extremely light, yet viable infections. It could be argued that we amplified sporozoites or remnants of abortive tissue stages from the blood of passerines, which would therefore be dead-end hosts of this parasite (Valkiūnas *et al.* 2009). However, this explanation will remain tentative before such parasite forms are observed in the blood of birds by microscopy. In fact, the PCR positives that scored the lowest detectable gametocytaemia in our study (one gametocyte in 10,000 erythrocytes) had qPCR estimates of parasitaemia that overlapped the values of birds without visible gametocytes. On the other hand, if sporozoites and remnants of abortive tissue were so easily detected in field samples, we should have retrieved a higher diversity of parasites from most bird species, which did not happen making this scenario questionable. Nevertheless, the possibility that PCR may sometimes amplify abortive infections needs to always be considered in studies of avian blood parasites. These dead-end infections may play an important ecological role if they harm the host (Olias et al. 2011) or represent near-successful cases of parasite host switching. From an epidemiological perspective, however, abortive infections would not contribute to transmission beyond limiting parasite spread through the invasion of incompatible hosts (Ostfeld & Keesing 2012).

Our study shows that neither molecular nor microscopy techniques alone can provide a complete picture of the host range and life cycle of parasites, and emphasizes the importance of using both methods in studies of haemosporidian infections (Valkiūnas *et al.* 2014). In order to increase the reliability of host-parasite interactions that can be inferred from the data contained in permanent repositories, we recommend researchers to state which parasite stages were confirmed by microscopy of host tissues for each genetic lineage uploaded in GenBank or MalAvi. This phenomenon may also be true for host specificity studies in other parasite systems, and we recommend combining molecular and morphological techniques when possible to reveal the real host specificity of parasites.

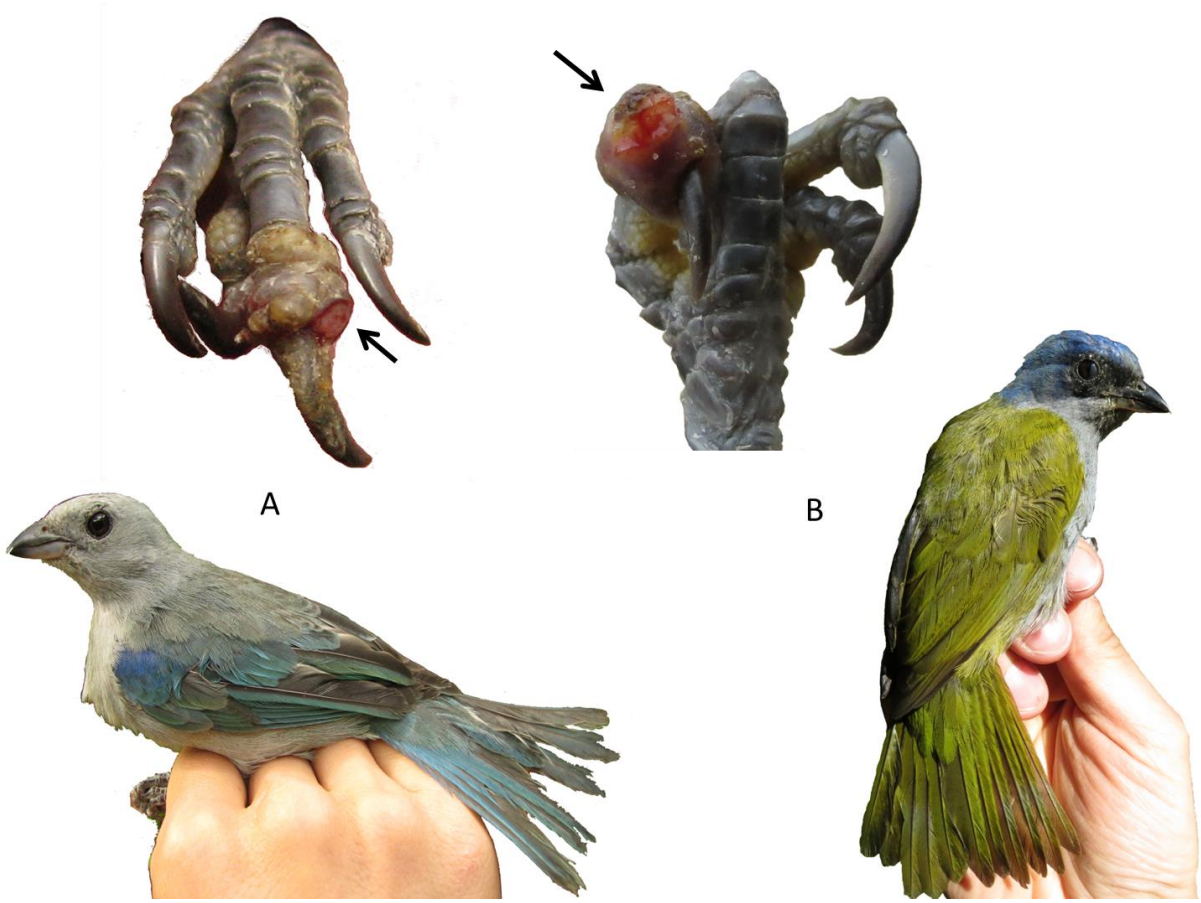
In summary, the *Haemoproteus* lineages we found in hummingbirds are shared with other bird families, which fits to the expectations of the niche breadth hypothesis to explain the evolution of generalist parasites through ecological fitting (Hellgren et al. 2009). In addition, the positive relationship between infection intensity and prevalence provides evidence for the existence of a positive abundance-occupancy relationship for *H. witti*, a pattern that remains underexplored at the intraspecific level for most parasites (Poulin 1999). Finally, our combination of molecular and

microscopy methods opens a debate of how to measure host specificity accurately in these parasites, an issue that will require further research to be solved. *Haemoproteus witti* is a generalist by its detection by PCR in many different species, but a hummingbird specialist by gametocytaemia. Because gametocytes are essential for haemosporidian transmission, this finding is challenging the current concept of host specialization in avian blood parasites. This may have implications in our understanding of the epidemiology and disease dynamics of blood parasites in wild bird populations and other host-parasite systems, and emphasises the importance of using several parasite detection techniques when studying local and global patterns of host specificity.

White-crowned Manakin

(*Dixiphia pipra*)

Chapter 3: The Biological Background of a Recurrently Emerging Infectious Disease: Prevalence, Diversity and Host Specificity of *Avipoxvirus* in Wild Neotropical Birds.



This chapter is based on the manuscript: **Moens M.A.J.**, Pérez-Tris J., Milá B. & Benítez Rico L. 2016. The Biological Background of a Recurrently Emerging Infectious Disease: Prevalence, Diversity and Host Specificity of *Avipoxvirus* in Wild Neotropical Birds. Under Review.

The Biological Background of a Recurrently Emerging Infectious Disease: Prevalence, Diversity and Host Specificity of Avipoxvirus in Wild Neotropical Birds.

Michaël A. J. Moens, Javier Pérez-Tris, Borja Milá & Laura Benítez

Abstract

Understanding which factors promote disease emergence and transmission remains a major challenge of epidemiology. A problem with research on emerging diseases is that we seldom know to what extent pathogens circulate in natural populations before emergence is already occurring. Moreover, it is critical to determine which pathogen attributes are key to predict their emergence potential. From this perspective, we analyzed the prevalence, host specificity and evolutionary relationships of *Avipoxvirus* causing skin lesions in birds in two megadiverse and unexplored geographical regions of South America: an elevational gradient in the South Ecuadorian Andes, and a lowland Amazon rainforest in French Guiana. In French Guiana at 200 m, *Avipoxvirus* prevalence was 0 % (n=307, 64 bird species). In Ecuador, prevalence was 0.3 % (n=941, 135 bird species), with cases spanning the range of elevations between 1500 and 2500 m. These were caused by two newly described strains, one of which belonged to an American clade of *Avipoxvirus* shared by different bird families, and another one closely related to a strain recovered from a different family of birds in Madeira. Our study at the community level shows that distantly related *Avipoxvirus* strains circulate at very low prevalence in continental tropical South America. An analysis of the host specificity and geographic distribution of all *Avipoxvirus* strains known worldwide finds that these viruses are usually host generalists (particularly those in the fowlpox clade), and are interchanged on a global scale. When *Avipoxvirus* assemblages are composed of generalist strains with different ancestry and widespread distribution, this combination of characters may make these typically scarce viruses perfect candidates to emerge under favorable ecological conditions.

Keywords: *Avipoxvirus*, generalist, host specificity, prevalence, specialist, South America

Introduction

The emergence of wildlife diseases depends on the combination of various factors operating at different scales, such as the coevolutionary history of hosts and pathogens, climate, or host community composition (Jones *et al.* 2008; Keesing *et al.* 2010). While much research has focused on understanding emerging infectious diseases when they already cause trouble to biodiversity, fewer studies have explored how pathogens circulate in natural populations before they become an imminent threat. A problem with the understanding of emerging diseases is that we seldom know the background biology of emergent pathogens, because the interest to study them arises after they emerged. From this perspective, studying the diversity and prevalence of circulating pathogens and the evolutionary relationships between these and their hosts is a critical issue (Fuller *et al.* 2012).

Viruses of the genus *Avipoxvirus* are good models to test ecological and evolutionary hypotheses as they are diverse and they can affect a broad diversity of bird species worldwide. They cause avian pox disease which is of extreme concern for the conservation of various bird species (Wikelski *et al.* 2004; Atkinson & Lapointe 2009; Carrete *et al.* 2009). These are linear double stranded DNA viruses with a large genome of up to 300 kilobases which are primarily transmitted by mosquitoes and sometimes through host body contact, contaminated surfaces, or aerosols (Tulman *et al.* 2004; Jarmin *et al.* 2006). They are represented by two main disease forms (van Riper III & Forrester 2007). The more common cutaneous form causes nodular lesions on featherless areas like feet, legs and areas surrounding the bill and eyes (Parker *et al.* 2011). The diphtheritic form affects the upper respiratory and digestive tracts and causes higher mortalities (Bolte, Meurer & Kaleta 1999; Offerman *et al.* 2013). To date, more than 60 unique strains have been detected in more than 278 avian species (Jarmin *et al.* 2006; van Riper III & Forrester 2007; Gyuranecz *et al.* 2013) and 10 *Avipoxvirus* species were recognized by the International Committee on Taxonomy of Viruses by early 2015 (King *et al.* 2012); http://www.ictvonline.org/). Phylogenies based on P4b core protein and DNA polymerase genes have revealed three major clades, which are the canarypox, the fowlpox and the psittacinepox clade (Jarmin *et al.* 2006; Manarolla *et al.* 2010; Gyuranecz *et al.* 2013).

Despite their importance as potential threats for bird diversity, prevalence patterns of these viruses are poorly understood. The great majority of research on *Avipoxvirus* epidemiology in wild birds has focused on emergence events in island endemics, while virtually no data are available on how *Avipoxvirus* circulates among most bird communities. Low prevalence seems to be the rule in temperate areas, ranging between 0.5 and 1.5% in most studies conducted to date (van Riper III & Forrester 2007). However, in some cases prevalence can reach 10-50% in certain host species (Davidson, Kellogg & Doster 1980; Mcclure 1989; Lawson *et al.* 2012). The highest prevalence values have been reported on oceanic islands such as Hawaii, Macaronesia and Galapagos (Vargas 1987; van Riper III, van Riper & Hansen 2002; Medina, Ramírez & Hernández 2004; Wikelski *et al.*

2004; Smits *et al.* 2005; Illera, Emerson & Richardson 2008; Carrete *et al.* 2009). On the slopes of Hawaiian volcanoes, maximum transmission occurs below 1500 meters, creating safe refugia for birds at higher elevations (van Riper III *et al.* 2002; Atkinson & Lapointe 2009). Similarly, in the Galapagos Islands higher prevalence is found in the lowlands than in the highlands (Vargas 1987; Kleindorfer & Dudaniec 2006).

Host specificity and dispersal potential of disease agents are also important characteristics, with generalist and easily transportable pathogens being good candidates to invade new hosts (Ewen *et al.* 2012). The host spectrum of *Avipoxvirus* seems to be broad (Haller *et al.* 2014). However, no quantitative analyses have been performed to study if there is variation in host specificity of *Avipoxvirus* strains among its major clades. Moreover, the current distribution of *Avipoxvirus* strains, and how it has changed during evolutionary history as a consequence of virus interchange among geographic regions is even more poorly understood. Filling these gaps in our knowledge would provide valuable insight into the relative importance of geography, climate and host diversity on *Avipoxvirus* transmission.

Here we aimed to analyze the prevalence, diversity and host specificity of *Avipoxvirus* in two megadiverse avifaunas in South America: an elevation gradient in the South Ecuadorian Andes, and a lowland rainforest in French Guiana. We conceived a community-level exploration as the best approach to understanding the pattern of variation in prevalence and host specificity of circulating virus strains, while contributing information for the first time on the diversity and prevalence of *Avipoxvirus* in the Neotropics. In order to understand the processes that structure *Avipoxvirus* assemblages, we analyzed the evolutionary relationships of Neotropical strains, aiming at identifying possible cases of virus transport or local diversification events. We then expanded our focus to the global diversity of *Avipoxvirus*, analyzing the host specificity and distribution of all known strains in order to understand their capacity to shift host species or location of transmission during evolutionary time. By spanning from local communities to global patterns of host-virus coevolution, our study aims to provide insight on how *Avipoxvirus* circulates in wild bird communities and what makes these viruses especially prone to cause disease emergence.

Material and methods

Field methods

The sampling was conducted at two sites in Ecuador and French Guiana. In Ecuador we captured birds (n = 941) using mist nets along an elevational gradient on the western slopes of the eastern cordillera in the Podocarpus National Park at four different altitudes (1500 meters above sea level (masl), 4°15'S, 79°13'W); 2000 masl, 4°14'S, 79°10'W; 2500 masl,4°23' S, 79°08'W; and 3000 masl, 4°23'S, 79°07'W) during five consecutive months (June-November, 2012), which started during the

rainy season and ended in the dry season. The sites varied from dry tropical forest to cloud forest, elfin forest and highland páramo. In French Guiana we carried out our study in a lowland Amazonian rainforest at the Nouragues reserve (4°05'N, 52°40'W), where we captured birds (n = 307) both in the Pararé and Inselberg research stations (located 8 km appart at 200 masl) during five weeks (March-April 2014), which was the transition between dry and rainy season. Birds were captured using 15 mist nets (12 m long × 2.5 m high, 25-mm mesh) and identified according to Ridgely & Greenfield (2006), Restall et al. (2007) and the checklist of the South American Classification Committee (Remsen *et al.* 2016). We took standard body measurements on all captured birds, and carefully inspected every individual for the presence of cutaneous lesions on and around featherless areas. When present, we collected a small part of the lesion by excision of a biopsy to maximize detectability of viruses by PCR from tissue DNA (Williams *et al.* 2014), and stored it in sterile tubes with absolute ethanol at ambient temperature. When symptomatic birds were handled, sterile gloves and scalpels were changed to avoid possible cross-contamination. Only one bird showed a small amount of bleeding on the lesion which was immediately stelped with styptic powder (KwikStop, Gimborn pet specialities, Atlanta, USA). For ethical reasons we preferred not to use analgesics or anestetics because this would require administration of chemicals with unknown side effects for the studied species, a method which could be more invasive. In addition, the sampled birds never showed abnormal behaviour compared to other birds. Once processed, birds were marked with a numbered aluminum ring to avoid repetition, photographed, and released unharmed at the site of capture.

Laboratory methods

The individual lesion samples were weighted, homogenized by mechanical force using a sterile plastic crusher, and total DNA was extracted with a standard phenol-chloroform-isoamyl alcohol protocol followed by isopropanol precipitation (Pérez-Tris *et al.* 2011). DNA extracts were controlled for sample quality on a 2% agarose gel, and DNA concentration was measured with a Nanodrop ND100 system (Nanodrop Technologies, ThermoScientific, Wilmington, DE) and stored at -20 °C. Samples were first tested for possible PCR inhibition by amplifying a fragment of the bird cytochrome *b* gene (Pérez-Tris *et al.* 2011) as a control, and all six tissue samples produced positive amplification. Then a multiplex PCR designed for the combined detection of *Avipoxvirus* and *Papillomavirus* was performed under conditions described by Pérez-Tris et al. (2011). This was done with a total reaction volume of 25 µl with 5 µl of a 10^{-1} dilution of DNA extract, 50 pmol/ml of each primer, 0.8 mM of each deoxynucleotide triphosphate, 4.0 mM of MgCl2, 0.125 µl of AmpliTaq DNA polymerase, and 2.5µl of 10× buffer solution (Applied Biosystems, Warrington, UK) under the following PCR conditions: 3 min of initial denaturation (95 °C), 45 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, followed by a final extension step at 72°C for 5 min (Pérez-Tris *et al.* 2011). None of the samples tested positive for *Papillomavirus*. Samples which tested positive for *Avipoxvirus* were visualized on a 2% agarose gel by the appearance of a DNA fragment of approximately 250 bp, and

were further analyzed by amplifying 448 bp of the P4b core protein gene (Pérez-Tris *et al.* 2011). This PCR was performed under the same conditions as the multiplex PCR and products were visualized on a 2% agarose gel stained with ethidium bromide. Positive samples were sequenced by Macrogen (Netherlands) and were compared with known P4b sequences available from GenBank.

We computed prevalence of *Avipoxvirus* as the proportion of birds with PCR-positive lesions in the whole population. We analyzed if *Avipoxvirus* infections were concentrated in certain bird genera or families by assigning a species rank to every host species (based on their relative capture rates) and mapping the distribution of detection probability of at least one infection among species sampled in Ecuador (ranked from highest to lowest), based on the observed number of infections.

Phylogenetic and host-specificity analyses

All sequences of known P4b strains of *Avipoxvirus* were downloaded from GenBank (last accessed: 14 December 2014) and manually aligned using Bioedit (Hall 1999). Our initial database contained 316 P4b sequences from which we extracted 66 unique strains, including the ones found in this study. We inferred a phylogenetic tree of P4b sequences by means of Bayesian analysis with the BEAST 2.0 software (Bouckaert *et al.* 2014) using unique P4b strains from this study and the ones found in the literature and Genbank, applying the most appropriate substitution model according to the Bayesian Information Criterion implemented in MEGA 5.2 (Tamura *et al.* 2011): GTR + G + I. We specified the parameters for the BEAST run in BEAUTI 2.0 (Bouckaert *et al.* 2014), and MCMC chains (Markov Chain Monte Carlo) were run for 10^9 generations, sampling every 100.000 trees. A Yule speciation prior and a strict clock model were used as our data could not reject this model based on the histogram of ucdl.stdev values in Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer/). Estimated sample sizes were all higher than 200. The 10.000 resulting trees were summarized with TreeAnnotator v2.1.2 (http://beast.bio.ed.ac.uk/treeannotator) and the phylogeny with the posterior probabilities of the nodes was displayed in Mesquite 2.75 (Maddison & Maddison 2011) for further analysis.

In order to calculate the host specificity of every *Avipoxvirus* strain we computed the S^*_{TD} index (Hellgren *et al.* 2009), which measures a host range for each strain considering the diversity of host species and the taxonomic distance between host species and its variance (Poulin & Mouillot 2003; Hellgren *et al.* 2009). The S^*_{TD} index was calculated as follows:

$$S^*_{TD} = S_{TD} + \frac{s-1}{1 + Var{S_{TD}}^{s}}$$

$$S_{TD} = 2\frac{\sum\sum_{i<j}\omega_{ij}}{s(s-1)}$$

$$VarS_{TD} = \frac{\sum\sum_{i\neq j}\left(\omega_{ij} - S_{TD}\right)^2}{s(s-1)}$$

where *ωij* is the taxonomic distance between host species *i* and *j* (i.e., how many taxonomical steps need to be taken to get to their most recent common ancestor) and *s* is the number of host species infected by the virus (Hellgren *et al.* 2009). Using the global S^*_{TD} values calculated for each strain, we reconstructed the ancestral states of host specificity through the phylogeny using the trace character history option (parsimony ancestral state method) in Mesquite 2.75. For a better visualization of the resulting tree, we sorted the global S^*_{TD}values for host specificity in five categories, namely strains infecting only one host species (1), various species of the same genus (2), or species of different genera (3), families (4) or orders (5). Then, we analyzed the geographical distribution of each strain by performing a Blast on GenBank (Access date: 9 September 2015) and counted the number of continents where each strain was detected in order to test if closely related strains were found in the same geographical areas.

Results

A total of 941 birds of 135 species and 307 birds of 64 species were screened for cutaneous lesions in Ecuador in 2012 and French Guiana in 2014, respectively (S1 and S2 appendices). In Ecuador, biopsies were taken from six individuals with apparent cutaneous lesions belonging to six different bird species (Table 1, S1 Fig). Not all lesions looked like the typical warts caused by *Avipoxvirus*, but all of them were collected to avoid losing potential cases of infection. All individuals sampled in French Guiana were free of cutaneous lesions (S2 Appendix). The multiplex PCR performed on lesion DNA was negative for *Papillomavirus* infections but three individuals were positive for *Avipoxvirus* (prevalence = 0.3 %). We identified two *Avipoxvirus* strains with the P4b-specific PCR in three different bird species. One strain (THREPI01) was found at 1500 m in the blue-gray tanager (*Thraupis episcopus*) and the other one (THRCYA01) was detected in the glossy-backed trush (*Turdus serranus*) and the blue-capped tanager (*Thraupis cyanocephala*) at 2000 m and 2500 m, respectively (Table 1, S1 Fig). The first strain was detected during the end of the rainy season in June 2016, while THRCYA01 was found in September and October during the dry season. Both strains belonged to the canarypox clade (Fig 1). THREPI01 is closely related to a strain found in Berthelot's pipit (*Anthus berthelotii*) on the island of Santo Porto in the Madeira archipelago (Illera *et al.* 2008), with which it shares 99% identity (Fig 1). THRCYA01 belongs to a clade of strains which infect the Northern Bobwhite (*Collinus virginianus*) in Arizona and experimentally infected canaries (*Serinus canaria*) and apanane honeycreepers (*Himatione sanguinea*) in Hawaii (Gyuranecz *et al.* 2013). New sequences were uploaded to Genbank (Table 1). From the six collected lesions we could successfully amplify *Avipoxvirus* DNA in three bird species (bold), resulting in two new strains. None of the samples scored positive for *Papillomavirus.*

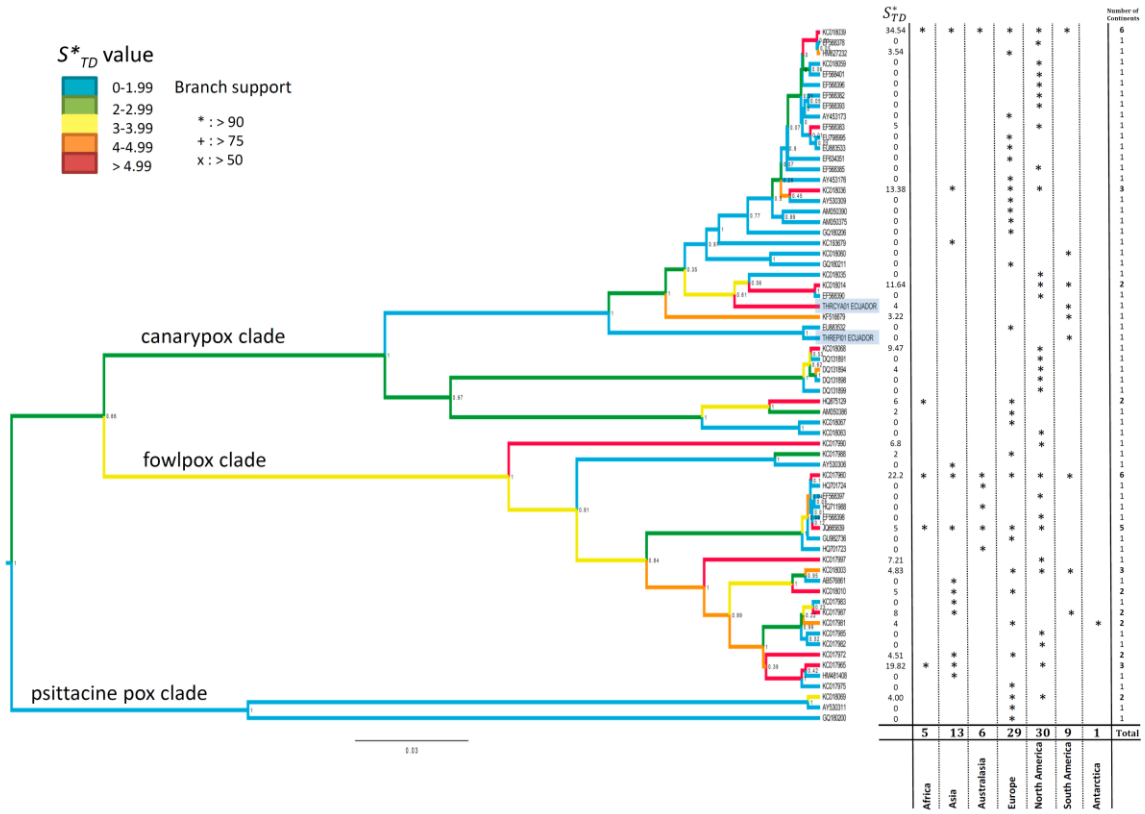


Fig 1: Bayesian phylogeny based on known P4b sequences of *Avipoxvirus* strains worldwide and the strains found in this study. The evolutionary history of host specificity of *Avipoxvirus* strains was calculated with a quantitative approach, in which each branch tip scores the global S^*_{TD} value (host-specificity index) of its lineage. We traced the evolution of host specificity along the phylogeny. For simplicity, S^*_{TD} values have been divided into five categories: zero representing strains infecting a single host species (blue branches); 2-2.99 (two host species of the same genus, green); 3-3.99 (two host genera, yellow); 4-4.99 (two host families, orange); >4.99 (two or more host orders, red). The new strains found in this study are marked in grey. The geographical distribution of all strains from the canarypox clade, the fowlpox clade and the psittacine pox clade are shown, indicating strains from the fowlpox clade are the most widely distributed across continents. Strains found in more than one continent are highlighted in bold.

Table 1: List of birds which presented pox-like lesions that were sampled for molecular analysis.

Bird Species	**Altitude**	**Multiplex PCR *Avipoxvirus***	**Multiplex PCR *Papillomavirus***	**P4b PCR**	**Strain**	**Genbank accession nr.**
Thraupis episcopus	1500	Positive	Negative	Positive	THREPI01	KU356758
Arremon torquatus	2000	Negative	Negative	Negative		
Myiothlypis coronata	2000	Negative	Negative	Negative		
Turdus serranus	2000	Positive	Negative	Positive	THRCYA01	KU356759
Thraupis cyanocephala	2500	Positive	Negative	Positive	THRCYA01	KU356760
Mionectes striaticollis	3000	Negative	Negative	Negative		

The probability of showing at least one *Avipoxvirus* infection (based on the random distribution of the observed infections among birds and their relative capture rates) was 0.035, 0.016 and 0.013 for the infected species: glossy-backed trush, blue-capped tanager and blue-gray tanager, which were 1st, 2nd

and 3rd in the rank of candidate species to score infections by chance, respectively (Fig 2). Taking these results a posteriori, the probability that the two species in the genus *Thraupis* scored two out of three observed infections by chance alone was p = 0.00021, revealing that birds of the genus *Thraupis* hold more infections than would be expected by chance (Fig 2).

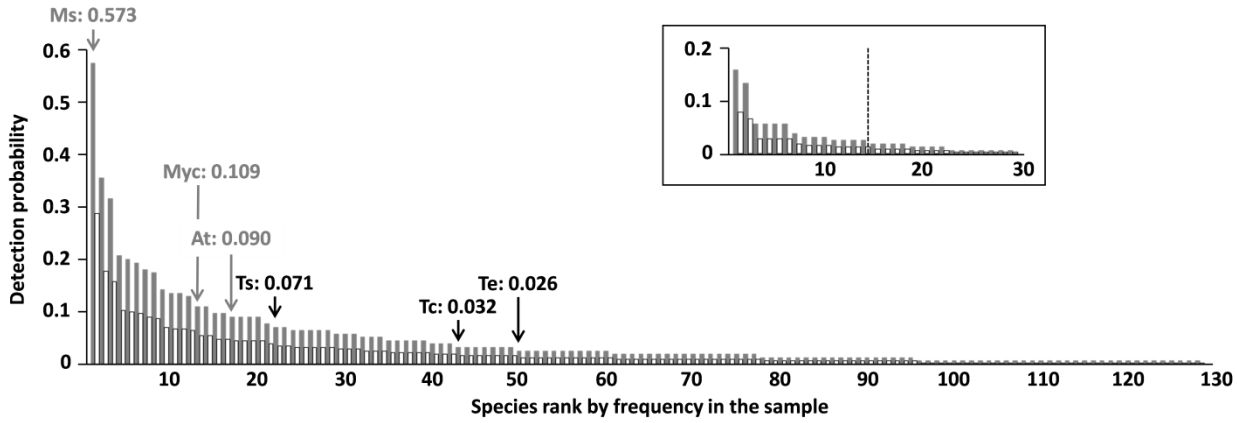


Fig 2: Distribution of the probability of detection of at least one infection among species sampled in Ecuador (ranked from highest to lowest), based on the observed number of infections and relative capture rates. Grey bars correspond to probabilities derived from six observations of birds with pox-like skin lesions, and open bars to those derived from infections confirmed by PCR. The arrows identify the expected rank of detection probability of the species which were found infected, either by pox-like skin lesions alone (in grey) or by lesions and PCR (in black). The numbers indicate the probability of a species scoring at least one skin lesion (the probability of scoring a PCR positive is half this value). Species acronyms stand for *Thraupis episcopus* (Te), *Arremon torquatus* (At), *Myothlypis coronata* (Myc), *Turdus serranus* (Ts), *Thraupis cyanocephala* (Tc) and *Mionectes striaticollis* (Ms). The inset shows the same data for our sample from French Guiana, based on a community-level prevalence equal to the one observed in Ecuador (in the absence of a better estimate).

Our analysis of S^*_{TD} values indicates a heterogeneous distribution of host specificity among *Avipoxvirus* strains, with the canarypox clade being generally more host specific than the fowlpox clade (Fig. 1). The canarypox clade diversified faster and yielded greater diversity than the fowlpox clade, with exception of a small subclade of generalist canarypoxviruses (Fig. 1). Four strains of the canarypox clade were found in at least two different continents while eight strains of fowlpox were found in more than one continent (Fig. 1). Closely related viruses showed distant geographic distributions which indicates a global interchange of these viruses, especially in the fowlpox clade (Fig. 1).

Discussion

We found very low prevalence of cutaneous lesions caused by *Avipoxvirus* in two Neotropical regions located in the Ecuadorian Andes and the lowland Amazon of French Guiana. It is interesting that our extensive sampling of a wide variety of passerines and non passerines did not detect more *Avipoxvirus* infections. This suggests that *Avipoxvirus* circulates at very low prevalence at the community level in these environments. Still, we would need long-term monitoring to detect the variation of prevalence of *Avipoxvirus* in these bird communities. However, sampling during one year would represent the

normal prevalence patterns rather than the extremes. To our knowledge, this is the first study analyzing *Avipoxvirus* prevalence and diversity in wild bird communities in continental South America and the tropics. Most *Avipoxvirus* research emphasizes case studies where *Avipoxvirus* already is an emerging disease in specific bird species, while much less is known about its circulation at the bird community level before disease outbreak. This study thus provides insight on the prevalence of *Avipoxvirus* in neotropical forests, which is estimated as very low. A relevant comparison would be the Galapagos Islands, where *Avipoxvirus* has been introduced and produced a high prevalence in certain endemic species (Parker *et al.* 2011). In a Galapagos mockingbird (*Mimus parvulus*) population, a prevalence of *Avipoxvirus* of 28 % and 7 % was detected in juveniles and adults, respectively (Vargas 1987). In a study on Galapagos finches, 6-13 % prevalence of *Avipoxvirus* infections was found in 2009 (Zylberberg *et al.* 2012) while Kleindorfer et al. (2005) found 7 % of *Avipoxvirus* lesions in 10 endemic finch species.

We can be confident that we did not miss many cases of cutaneous lesions caused by *Avipoxvirus* infection since we exhaustively inspected all birds and sampled all suspicious lesions, even if they did not show the typical symptoms of *Avipoxvirus*. This is a possible reason why three tissue samples resulted negative after PCR analysis, since they represented a more atypical lesion. However, the time frame when infections are detectable with our method is likely shorter than the period of infection of the bird. Infected birds may be able to transmit the virus before the lesion becomes visible. In addition, morbidity caused by the infection could to some extent reduce the probability of capturing infected birds if these are less active. These biases may lead to prevalence being underestimated in studies of wildlife diseases.

The low prevalence of *Avipoxvirus* in this study is probably not due to high altitude of the study area, as it happens in other regions where *Avipoxvirus* is hardly transmitted where mosquitoes are scarce due to altitude (van Riper III & Forrester 2007). In fact, we found infections at relatively high elevation (but none at the low elevation site in French Guiana), to the point that we report the maximum elevation at which *Avipoxvirus* has been recorded in the wild (2500 m). Maximum elevation recorded in Hawaii was 1830 m (2% prevalence; Atkinson et al., 2005), where the transmission of *Avipoxvirus* is limited to lower areas due to the distribution of their main vector, *Culex quinquefasciatus* (Ahumada, Lapointe & Samuel 2004; Aruch *et al.* 2007). It has been shown that prevalence of *Avipoxvirus* is related to vector distributions and climatic conditions on a local scale (Aruch *et al.* 2007; Young & Vanderwerf 2008). Further research on the vectors of *Avipoxvirus* in the Andes and other mountain ranges is therefore needed to understand the biogeographical limits in the distribution of these viruses. Furthermore, in areas where *Avipoxvirus* is endemic, prevalence seems to be lower than in naïve host populations (van Riper III *et al.* 2002; van Riper III & Forrester 2007). Since our sampling sites are not isolated (compared to islands), it could be that *Avipoxvirus* is endemic here, and birds are less immunologically naïve.

Other ecological factors, such as bird congregation where humans provide food, sets an ideal scenario for increasing intra-specific and inter-specific contact which could enhance pathogen transmission (Carrete *et al.* 2009; Giraudeau *et al.* 2014). In this context, it is noticeable that the bird genus *Thraupis* scored two of the three infections, given the poor sampling of this genus (our sample includes five individuals of *T. cyanocephala* and four of *T. episcopus*). The probability that two species of *Thraupis* score at least one infection is therefore very low (P = 0.00021), although this estimate cannot be interpreted in terms of statistical significance given that the observation was made without *a priori* predictions on the distribution of infections across species. In any case, this observation points to tanagers as potentially relevant reservoirs of *Avipoxvirus* in the Andes. Indeed, the genus *Thraupis* could be particularly important to understand the epidemiology of *Avipoxvirus* in the Northern Andes and this warrants future research. The blue-gray tanager is associated with human activity and can be found around farms, which could promote the transmission of *Avipoxvirus* in this species. The glossy-backed trush also seems to be a good candidate to host these viruses, given that other *Avipoxvirus* strains have been retrieved from the genus *Turdus* in other areas of the world (Ha *et al.* 2013). In summary, we found *Avipoxvirus* strains in birds which have a suitable ecology and ancestry to host avipoxviruses, indicating that these bird species may not have scored the infections observed in our sample by chance alone.

Considering the low prevalence of *Avipoxvirus* here, it is remarkable that we found the same strain in two distinct species at two different locations separated both by distance and altitude. This is evidence of low diversity of *Avipoxvirus* in the Andean area. Both strains found in Ecuador are new and add diversity to the canarypox clade. Strain THREPI01 is the most related to a virus detected in wild Berthelott's pipits on the Madeira archipelago, an observation which is remarkable considering the geographical distance, especially because this strain has not been detected in other species. TURSER01 belongs to a subclade of canarypox with more generalist strains, while THREPI01 has so far been retrieved only from a tanager in our study, so that little can be said for the moment about its host range. It is important to note that many strains are recovered only once in different bird species and studies at the host community level are scarce, which indicates we are only beginning to uncover the real diversity, distribution, and host specificity of *Avipoxvirus*. The canarypox clade is more diverse than the fowlpox clade and consists of more specialist strains, suggesting canarypox specialization may make it less prone to shift to different host orders.

Importantly, we highlight two key attributes which make *Avipoxvirus* a concern as a recurrently emerging pathogen. First, we found that *Avipoxvirus* strains are transmitted on a worldwide scale, particularly those belonging to the fowlpox clade, where some strains have been found in six different continents. Moreover, sister strains in both canary- and fowlpox clades are found in distant continents, which supports the idea that extensive host and distribution shifts have taken place in the *Avipoxvirus* genus. We thus found evidence of broad-scale interchange of viruses (close relatives found in

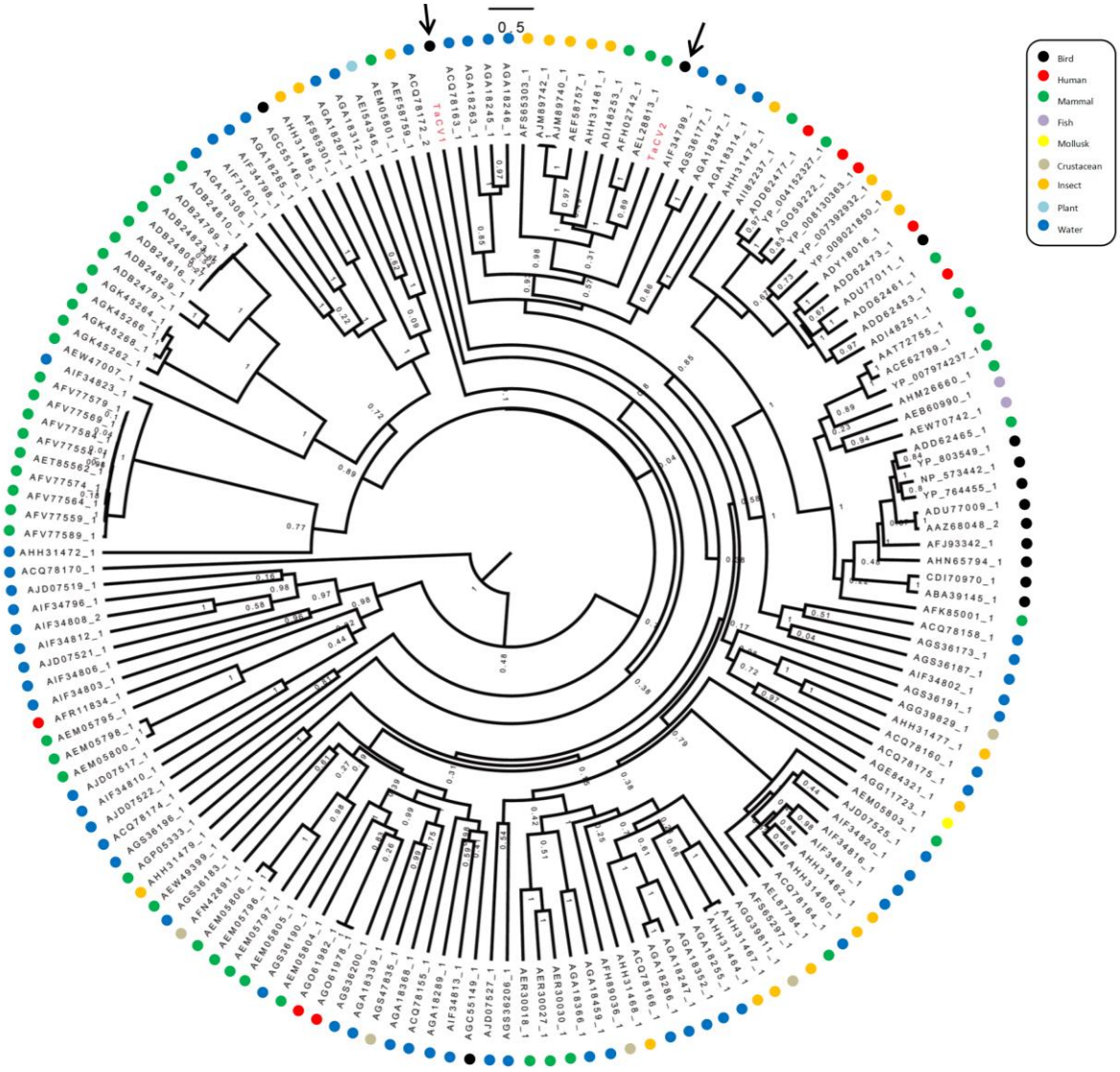
distantly located areas, such as THREPI01 and its sister strain on the Madeira islands) which did not involve change of bird order. This suggests that *Canarypoxvirus* may be free to disperse among geographic regions but remains restricted to passerine birds. Second, our quantitative analysis reveals the generalist nature of *Avipoxvirus* strains, particularly in the fowlpox clade. This feature makes the most generalist strains good candidates to emerge as a disease when introduced into new areas and host species (Ewen *et al.* 2012) and make them a priority of monitoring programs in the future in order to protect endemic, endangered and immunologically naive bird species. The introduction of generalist *Avipoxvirus* strains to island avifaunas such as in Hawaii and Galapagos (van Riper III *et al.* 2002; Parker *et al.* 2011) is a clear example which led to the emergence of disease on both archipelagos, and highlight the dispersal and host-shifting capacities of these viruses.

Taken together, our study supports the idea that *Avipoxvirus* circulates at a low prevalence and diversity at the community level in forest understory of the continental Neotropics, but due to their high dispersal capacity and low host specificity, they could spread to new host species when particular circumstances occur. The combination of naïve hosts with a short coevolutionary history with invasive parasites, adequate climatic conditions to maintain infective vector populations, and a reduced diversity of hosts, provide favorable conditions for the emergence of *Avipoxvirus* in new environments. In these circumstances new opportunities are also created to disperse on a large scale, for example through long-distance bird migration (Parker *et al.* 2011; Fuller *et al.* 2012). Extensive sampling of birds in the Neotropics and elsewhere is needed to understand the variation in host specificity and distribution of *Avipoxvirus* strains. This will also help to recognize the value of biodiversity in suppressing disease emergence and its effects on the transmission of *Avipoxvirus* in wild populations of birds.

Royal Flycatcher

(*Onychorhynchus coronatus*)

Chapter 4: Characterization and evolutionary relationships of two novel CRESS DNA viruses isolated from an *Avipoxvirus* lesion of a common bird in Ecuador.



This chapter is based on the manuscript: **Moens M.A.J.**, Pérez-Tris J., Cortey M. & L. Benítez. 2016. Characterization and evolutionary relationships of two novel CRESS DNA viruses isolated from an *Avipoxvirus* lesion of a common bird in Ecuador. Under review.

Characterization and evolutionary relationships of two novel CRESS DNA viruses isolated from an *Avipoxvirus* lesion of a common bird in Ecuador.

Michaël A. J. Moens, Javier Pérez-Tris, Martí Cortey & Laura Benítez

Abstract

The discovery of circular rep-encoding single stranded (CRESS) DNA viruses has aumented spectacularly over the past decade. They represent the smallest viruses known worldwide infecting a wide variety of invertebrates and vertebrates in different natural and human made environments. The extremely low similarity of nucleotide and protein sequences among different CRESS DNA genomes has challenged their classification. Moreover, the existence of putative capside proteins (Cap) remains difficult to demonstrate which is crucial to understand the structural properties of these viruses. Here we describe two new CRESS DNA viruses isolated from a cutaneous lesion, caused by *Avipoxvirus*, in a blue and gray tanager (*Thraupis episcopus*) of Southern Ecuador. Both viruses present a functional replication-associated protein (Rep) and one to two open reading frames (ORF) which represent a putative Cap protein. Within these putative Caps, we detected intrinsically disordered regions (IDR), putative protein binding regions, several motifs related to rolling circle replication and nuclear localization signals (NLS), providing further evidence of presumed Cap proteins. We describe a new characteristic IDR profile for CRESS DNA viruses. Despite being found on the same host lesion, both viruses show low similarity between each other (<60%) and other known CRESS DNA viruses. By analyzing evolutionary relationships within the CRESS DNA diversity, we show that host switching has been important during their evolutionary history. Additional sampling is needed to explore the possible pathogenic effects, prevalence and diversity (both phylogenetical and structural) of these viruses in nature.

Keywords: *Avipoxvirus*, CRESS DNA virus, Ecuador, Host Switching, Intrinsically Disordered Regions. Tanager

Introduction

Many CRESS DNA viruses have been discovered in a wide diversity of natural and human-made environments, such as estuaries (Dayaram *et al.* 2015), sewage oxidation ponds (Kraberger *et al.* 2015), oceans (Labonté & Suttle 2013) and in many animal groups spanning from arthropods to vertebrates, including humans (Rosario *et al.* 2015). These viruses are the smallest known viruses to infect eukaryotic organisms and evolve rapidly due to their high recombination and mutation rates (Duffy *et al.* 2008; Lefeuvre *et al.* 2009; Martin *et al.* 2011), which make them good candidates to develop into emerging pathogens (Rosario *et al.* 2012). Their genome is usually smaller than 6 kb and they use a rolling circle mechanism for genome replication, also relying on host cellular proteins for successful replication (Rosario *et al.* 2012). Many CRESS DNA viruses present novel genome organisations, including similarities with Rep proteins of other viruses. Still the function of other proteins such as Cap proteins needs to be explored in order to understand the mechanisms how these viruses replicate in a wide diversity of hosts.

In birds several ssDNA viruses have been detected, mainly belonging to the *Circoviridae* family (Todd 2004; Stewart *et al.* 2006; Todd *et al.* 2010). According to the International Committee on Taxonomy of viruses the family consists of the genus *Circovirus* and proposed *Cyclovirus*, with the *Gyrovirus* recently moved to the family of *Anelloviridae* (Biagini et al., 2014). A new study on circo-like viruses in mosquitoes suggest the evidence of a new genus *Krikovirus* inside the *Circoviridae* family (Garigliany *et al.* 2015). The genomes of this family typically have a circular ambisense organization consisting of two or more major ORFs, encoding a Rep and a Cap protein and a stemloop origin of replication appearing as a conserved nomamere motif (5'-NANTATTAC-3') in between the 5'-ends of both ORFs (Rosario *et al.* 2012). Avian circoviruses have been associated with a variety of illness symptoms such as immuno supression, delayed growth, feather disorders and developmental abnormalities (Todd 2000, 2004; Stewart *et al.* 2006). Based on the above these viruses are probably widespread and diverse, but they are poorly studied. Therefore, it is urgent to improve our knowledge of their phylogenetic and functional diversity, their ecology (how they interact with their hosts or other pathogens) and their biogeography. In this paper, we describe a singular yet informative case in which two new genomes of CRESS DNA viruses were isolated from an Avipox lesion of a common tropical bird, the blue-and-gray tanager (*Thraupis episcopus*). This bird was found infected with an *Avipoxvirus* strain which caused a cutaneous lesion on the foot, from which the two new genomes were isolated. Moreover we try to explore the structural function of novel putative capside proteins in order to understand the biological properties of these viruses. We will do this by investigating the presence of IDRs, which are regions within a protein that lack an ordered structured (He *et al.* 2009). These IDRs allow a protein to exist in different states depending on the substrate they interact with (Dunker *et al.* 2001) and could reveal the possible existence of Cap proteins in CRESS DNA viruses (Rosario *et al.* 2015). Within these IDRs we aim to detect the

presence of protein binding regions which are disordered in isolation but which can undergo disorder-to-order transition upon binding (Mészáros *et al.* 2009). Moreover we will try to indentify motifs in the amino acid sequences which support the position of a functional Cap-like proteins. Finally, we will analyse their evolutionary relationships within the known diversity of CRESS DNA viruses to explore to what extent host switching has been important during the evolutionary history of this megadiverse group of viruses.

Materials and Methods

The case reported here was sampled during a mist-netting campaign designed to investigate the prevalence of various avian pathogens in wild bird communities of the Ecuadorian Andes, which involved blood sampling and revision of possible virus infections. The list of screened pathogens included viruses causing skin lesions like Poxvirus and *Papillomavirus*. In all, we inspected 941 birds of 135 species, of which six showed cutaneous lesions on their feet compatible with virus infection. The lesions were weighted, homogenized by mechanical force using a sterile plastic crusher and viral DNA was extracted with a standard phenol-chloroform-isoamyl protocol followed by isopropanol precipitacion (Pérez-Tris et al., 2011). We extracted total DNA from blood samples with a standard ammonium acetate protocol (Green et al., 2012). DNA-extracts were controlled for sample quality on a agarose gel, were tested on nucleic acid concentration with a Nanodrop ND100 system (Nanodrop Technologies, ThermoScientific, Wilmington, DE) and stored at -20 °C. First a multiplex PCR designed for the combined detection of *Avipoxvirus* and *Papillomavirus* was performed under conditions described by Pérez-Tris et al. (2011). This was done with a total reaction volume of 25 µl with 50 pmol/ml of each primer, 0.8 mM of each deoxynucleotide triphospate, 4.0 mM of MgCl2, 0.125 µl AmpliTaq DNA polymerase and 2.5µl of Buffer solution 10x (Applied Biosystems, Warrington, UK) under the following PCR conditions: 3 min. of initial denaturation (95 °C), 45 cycles of 95°C for 1 min., 50°C for 1 min. and 72°C for 1 min., and a final extension step at 72°C for 5 min. (Pérez-Tris et al., 2011). Samples which tested positive for poxvirus were visualized on an 2 % agarose gel by the appearance of a DNA fragment of approximately 250 base pairs and were further analysed by amplifying part of the P4b core protein gene (Lee & Lee 1997). This PCR was performed under equal conditions as the multiplex PCR and products were visualized on an agarose gel stained with Gel Red. The positive sample for Avipox was sequenced by Macrogen (Netherlands) and the sequence was compared with known P4b-isolates on Genbank.

Next we performed a rolling circle amplification (RCA) in order to amplify circular DNA genomes from the lesion (Dean *et al.* 2001; Johne *et al.* 2009). The RCA was initially performed to amplify *Papillomavirus* DNA (Rector *et al.* 2004), as the lesions also could have been caused by this virus. Random hexamer primer gets annealed to the ssDNA molecule and the phage phi29 polymerase adds nucleotides in the 5’ to 3’ direction complementary to the template strand until the primer strands

again, after which it displaces the new synthesized strand and continues with DNA synthesis for several rounds (Johne *et al.* 2009). This isothermal amplification produces high quantities of single- or double stranded-DNA and the proofreading activity of this polymerase ensures high-fidelity DNA replication. DNA was amplified using multiple-primed RCA (Johne *et al.* 2009) with TempliphiTM 100 Amplification (GE Healthcare) following the manufacturer's instructions. Multiple-primed RCA amplifications were carried out with 0.5 µl of DNA in a total reaction volume of 10 µl. Out of six birds with lesions, three tested positive for Avipox, one of which also produced RCA products.

The RCA positive bird was captured the 22 of June 2012 near San Pedro de Vilcabamba (4°15'S, 79°13'W, 1800 m elevation), in a suburban mosaic of scrub and riverine vegetation on the Rio Chamba river banks . After the completion of the RCA reaction the product was digested with the EcoRI enzyme. We ran an agarose gel of 1.5 % stained with ethidium bromide to visualize the digested product. Four DNA fragments with different intensities were extracted from the agarose gel (QIAquick Gel Extraction Kit, QIAGEN, Germany) and cloned. The vector pUC19, cut with Hind III and processed with shrimp alkaline phosphatase (Roche Applied Sciences) to avoid re-ligation, was ligated to the RCA digested fragments in a total volume of 10 µl with the T4 DNA ligase (Roche Applied Sciences). One shot TOPO10 competent *E. coli* (Invitrogen) were transformed with the resulting plasmids. We extracted the plasmid DNA from recombinant clones with a QIAprep Miniprep Spin kit (Qiagen).

Next, succesful cloning products were sequenced. After a BLAST search on GenBank we detected nucleotide similarity with Rep proteins of other CRESS DNA viruses. We designed two sets of back-to-back primers located inside of two sequenced fragments correponding to both Rep proteins and two sets of conventional (non-overlapping) primers (Supplementary table 1). A standard PCR with both type of primers was performed on the RCA products with TaKaRa taq polymerase (TaKaRa Bio Inc, Otsu, Shiga, Japan), which can amplify fragments up to 15 kb. Aditionally, a PCR with back-to-back sets of primers was done on the original DNA extraction of the lesion (1µl). We ran an extra PCR on the extraction of the blood sample (2µl) of the infected bird to control if the infection can be detected in the blood. Both PCRs were run with the following conditions: 1 min. of initial denaturation (94 °C), 30 cycles of 98°C for 10 seconds, 43°C for 30 seconds and 68°C for 10 min., and a final extension step at 72°C for 10 min. A 2 % agarose gel was ran to visualize the PCR products. The multiple sequences obtained from all protocols were assembled into two distinct genomes using the DNAstar software (Madison, Wisconsin, USA). The genomes were compared to others by means of a BLAST search of the GenBank database. Putative ORFs were assigned by means of SMS ORF Finder (http://www.bioinformatics.org/sms2/orf_find.html). Secondary structures of the proteins were analysed by a web-based version of mfold (http://mfold.rit.albany.edu/?q=mfold/DNA-Folding-Form).

In order to study evidence of host switching and the phylogenetic position of both Rep proteins within the CRESS DNA diversity we performed a Bayesian analysis with BEAST 2.0 (Bouckaert *et al.* 2014). We decided to infer phylogenetic relationships of the Rep proteins since the amino acids are easily aligned compared to the other ORFS, which do not show many similarities between each other. Moreover, the Rep protein has been proven useful in deciphering evolutionary relationships in CRESS DNA viruses. We aligned amino acid sequences of the Rep protein of 159 CRESS DNA viruses, based on the highest similarities found in the BLAST searchin GenBank. We used the most appropriate substitution model for the Rep protein amino acid sequences according to the Bayesian Information Criterion implemented in MEGA 5.2 (Tamura *et al.* 2011): LG+G. We specified the parameters for the BEAST-run in BEAUTI 2.0 (Bouckaert *et al.* 2014) and Monte Carlo Markov Chains (MCMC's) were run for 10^9 generations, sampling every 100.000 trees. Traces were inspected for convergence with Tracer 1.5 (Rambaut & Drummond 2007). The 10.000 resulting trees were summarized with TreeAnnotator v2.1.2 (Rambaut & Drummond 2007) and the phylogenies with the posterior probabilities of the nodes were displayed in FigTree v1.4.2 (Maddison & Maddison 2011) for further analysis. We plotted the host group in which all CRESS DNA viruses were found to analyze if these viruses infect hosts of restricted or wide phylogenetic ancestry. In order to identify possible patterns of similarity of the Rep proteins of these viruses with different CRESS virus types already described in the literature, a pairwise identity matrix of the same Rep proteins and genomes was created with the sequence demarcation tool of the SDT software (Muhire, Varsani & Martin 2014). We used the same selection of 159 CRESS DNA genomes and their correspondent Rep proteins. All sequences were aligned with MUSCLE (Edgar 2004) within the SDT software.

We used the Tandem Repeat Finder-software to detect tandem repeats in both genomes (Benson 1999). In order to predict nuclear localization signals and DNA binding residues in the amino acid sequences of both genomes we used PredictNLS (Cokol, Nair & Rost 2000) and BindN (Wang & Brown 2006) respectively. We predicted intrinsically unstructured regions within the putative ORF's with the IUPred software (Dosztanyi *et al.* 2005). IUPred calculates a pairwise energy profile along the amino acid sequence, where values above 0.5 indicate disordered regions within the protein. Results were also compared with the DisProt VL3 disorder predictor (Obradovic *et al.* 2003; Sickmeier *et al.* 2007) and the PONDR-fit software which combines several predictors like VL3, VL2 and VLXT (Xue *et al.* 2010). Protein binding regions within the IDR's were predicted with the ANCHOR software (Dosztányi, Mészáros & Simon 2009). ANCHOR pursues to identify segments within disordered regions, which cannot form enough favorable intra-chain interactions to fold on their own and which are likely to gain stabilizing energy by interacting with a globular protein partner (Dosztányi *et al.* 2009). ANCHOR then generates a probability score profile of the amino acid residues, indicating the likelihood of the residue to be a part of a disordered region along the sequence (Dosztányi *et al.* 2009). Regions with scores above 0.5 indicate disordered binding regions. The novel

CRESS DNA viruses were uploaded to GenBank under the following accession numbers (*to be assigned*).

Results and Discussion

After the lesion was found positive for *Avipoxvirus*, a RCA was performed to detect a possible coinfection with *Papillomavirus* (Johne *et al.* 2009) which after digestion with EcoRI resulted various fragments (Fig. 1A). The lack of equimolar amounts of DNA suggested at least two different molecules, indicating the existence of more than one original virus (Fig. 1A). Four of these fragments were cloned and sequenced showing the existence of two fragments belonging to two very divergent Rep protein genes. The design of several sets of back-to-back (overlapping) and non-overlapping primers along the Rep genes and several walking primers allowed the complete sequenciation of two different virus genomes and confirmed their circular structure (Fig. 1B). We succesfully amplified one of both viruses with a PCR on the original lesion extract (Fig. 1C). The second virus was not amplified by PCR on the lesion extract (Fig. 1C), probably due to its low concentration. We did not amplify the viruses in the blood sample (Fig. 1C).

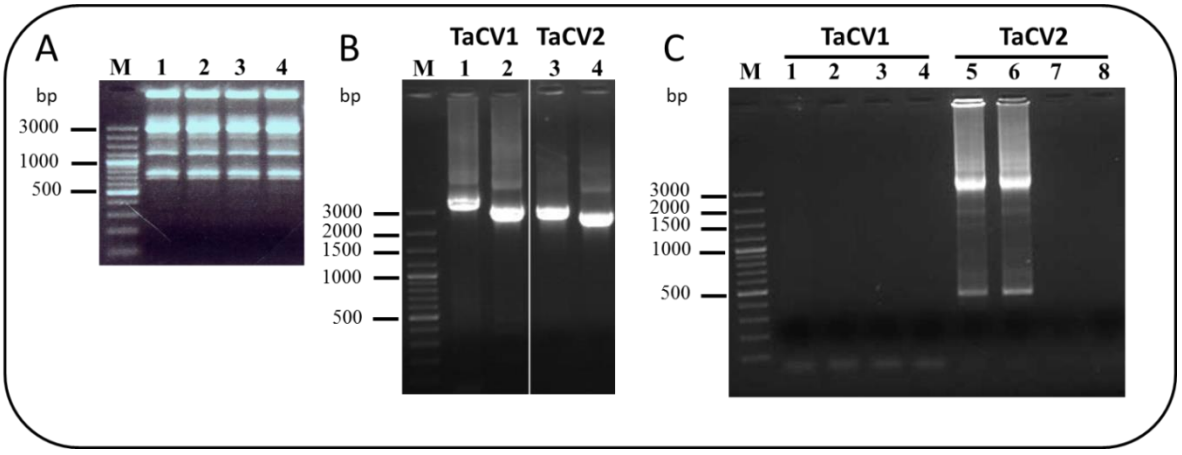


Figure 1: Agarose gels with amplified products: (A) EcoRI-RCA pattern obtained from four independent amplifications (1-4). The lack of equimolar amounts of DNA suggested at least two different molecules; (B) PCR amplification on RCA product using back-to-back primers CIR1000F1S/R1S (line 1), CIR3000F1S/R1S (line3) and conventional primers CIR1000F2/R2) (line 2), CIR3000F2/R2 (line 4); (C) Direct PCR amplification on the lesion DNA and blood sample using back-to-back primers CIR1000F1S/R1S (lines 1 to 4) and CIR3000F1S/R1S (lines 5 to 8). Lines 1, 2, 5 and 6 (amplification of 1µl of DNA extraction of lesion), line 3 and 7 (amplification of 2 µl of blood sample), lines 4 and 8 are negative controls. M: GeneRuler 1 kb DNA ladder Plus (ThermoFisher Scientific).

After all the analyses we succesfully assembled two CRESS DNA virus genomes from the *Avipoxvirus* lesion. We name them Tanager-associated CRESS DNA virus TaCV1 (3401 nt) and TaCV2 (2967 nt). TaCV1 showed an ambisense genome organization with a replication-associated protein (Rep) of 393 amino acids in sense direction and one open reading frame (ORF1) of 208 amino acids in the antisense direction (Fig. 2). TaCV2 showed an ambisense genome organization with two ORFs (ORF1, ORF2) in ambisense direction of 166 and 174 amino acids respectively and a

replication-associated protein (Rep) of 321 amino acids in sense direction (Fig. 2). TaCV1 had four EcoRI restriction sites while TaCV2 contained a single one, which is clearly represented in Fig. 1A. The top fragment corresponds to TaCV2 while the remaining fragments belong to TaCV1 (Fig. 1A). The intensity of the latter fragments is lower suggesting a smaller concentration of the inicial virus load for TaCV1 (Fig. 1A).

Two long noncoding A-T rich regions were located between ORF1 and the Rep protein in TaCV1 (978 b) and Rep and ORF1 in TaCV2 (874 b) where the origin of viral replication is placed, although circoviruses have tipically G-C rich noncoding regions which are usually shorter (Fig. 2 B and 2C). A putative hairpin (13/14 nt in TaCV1/TaCV2) coincident to the length described in circovirus plus a conserved nonanucleotide motif in a stem-loop structure, where rolling circle replication is iniciated in circular ssDNA replicons, was found in both genomes (Fig. 2 B and 2C). The TaCV1 nonanucleotide 5´-CAGTATTAC-3´ has been described in other avian circoviruses (AJ304456 and DQ172906 from GenBank) while the 9 bases motif of TaCV2 (5´-TAATACTAT-3´) is highly conserved in many cycloviruses (Delwart & Li 2012). Moreover, 3.5 units of tamdem direct repeats of a 26 nt sequence (TaCV1) and 2.7 units of a 35 nt (TaCV2), which usually are associated to promoter-enhancer activity, were located in both noncoding regions (Fig. 2 B and 2C). Adjacently to the hairpin structure in TaCV1, two tandem repeats of the sequence 5'-GGAGCCA-3', described as putative binding sites for the Rep protein (Phenix *et al.* 2001), were found at positions 15 and 23 and downstream at positions 148 and 180, near a secondary hairpin of unknown function (Fig. 2 B and 2C). Both genomes also presented several TATA boxes (motif TATAA) located in these non-coding regions (Fig. 2 B and 2C).

Interestingly both Rep proteins of both genomes showed low similarity (26% identity, 100% coverage) between each other. However, the Rep protein of TaCV1 showed higher similarity with published Rep proteins of Penguinpox (30%, 48% coverage), and the Rep protein of TaCV2 was more similar to Canarypox (61%, 88% coverage). The nucleotide identity of the Rep protein of TaCV2 even reaches 81 % identity (48% coverage of total Rep protein towards the N-terminal) with the Canarypox Rep gene. This suggests that it is probably a recombinant sequence, where the N and C terminal have distinct evolutionary origins, like it has been reported previously in ssDNA virus genomes (Krupovic *et al.* 2015). Recombination and reassortment among ssDNA viruses has not been studied in detail compared to dsDNA viruses and this warrants further research. CRESS DNA viruses can combine genes from different types of nucleic acids (RNA and DNA) and different functional domains of the virus genes can have origins in different virus families, which increases their capacity to exploit new niches and to switch hosts (Krupovic *et al.* 2015; Lefeuvre & Moriones 2015) but complicates their characterization and classification.

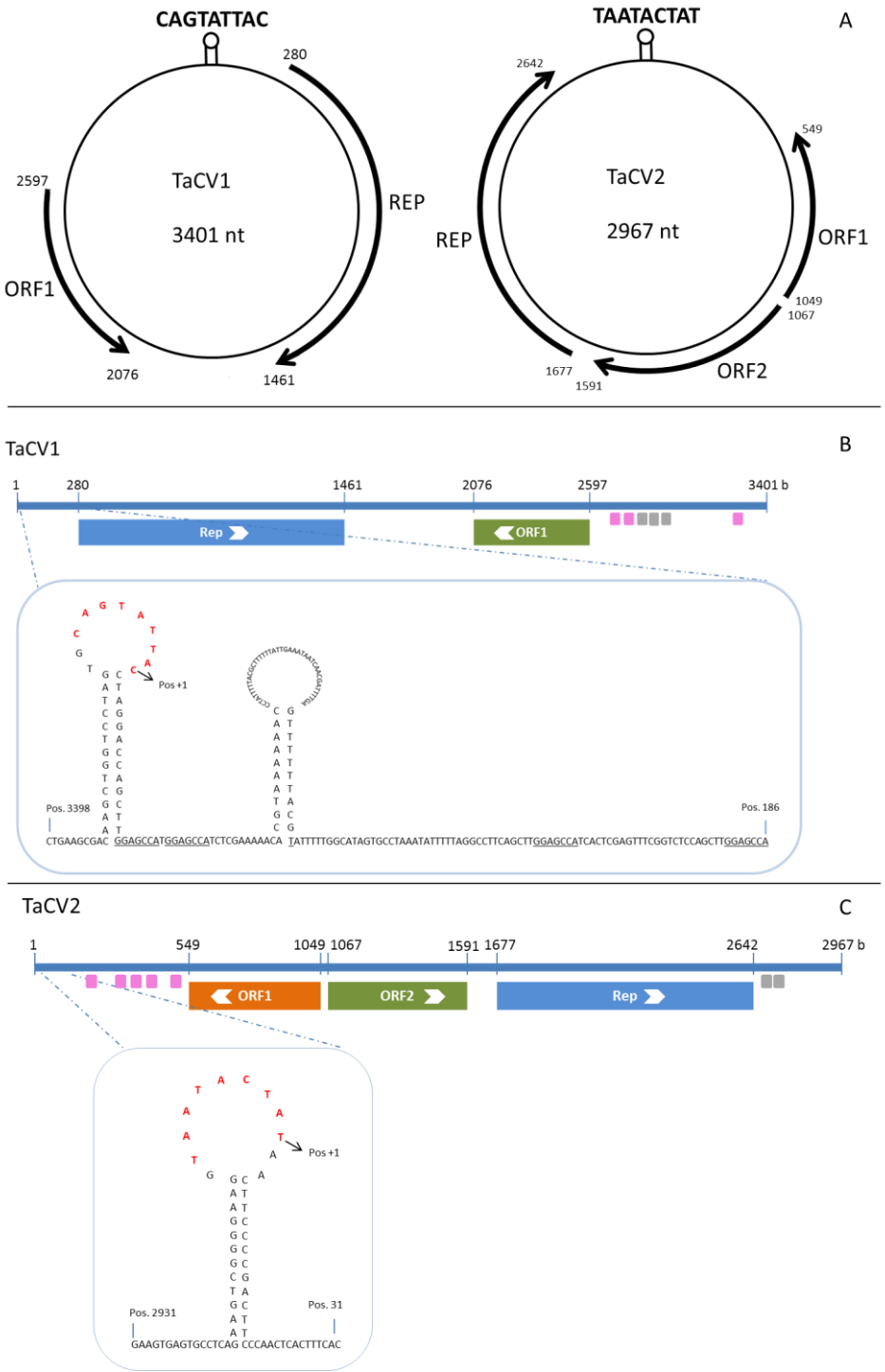


Figure 2: Genome organization of the two novel CRESS DNA viruses described in this study. (A) Circular structure of TaCV1 and TaCV2, lengths of the ORFs and location of the stem-loop structures on the origin of replication. (B) Detailed stem-loop structure of TaCV1, ORF orientation, tandem repeats of 26 nt (grey boxes) and TATA boxes (pink boxes). (C) Detailed stem-loop structure of TaCV2, ORF orientation, tandem repeats of 23 nt (grey boxes) and TATA boxes (pink boxes). Nonamers are marked in red and protein binding regions are underlined.

The pairwise identity matrix of the Rep proteins sequences shows that the Rep protein of TaCV1 shares 32% pairwise identity (100% coverage) with AFN42891.1 (*Acartia tonsa* copepod circovirus) while the Rep protein of TaCV2 shares 47% identity (100% coverage) with two Rep proteins

belonging to bat circoviruses (AEL28813, AFH02742) (Fig. 3). The genome SDT analysis shows that TaCV1 shares 64% pairwise identity with a bat circovirus from China (JN377580) and several uncultured marine viruses (JX904147, JX904605, JX904185, JX904344) while TaCV2 is 64% identical to bat guano *Circovirus* (HM228875) (Fig. 3). Curiously TaCV1 thus showed moderate similarity with CRESS DNA viruses associated with marine environments (Dunlap *et al.* 2013; Labonté & Suttle 2013) while TaCV2 is most similar to circoviruses associated with bats (Linlin *et al.* 2010).

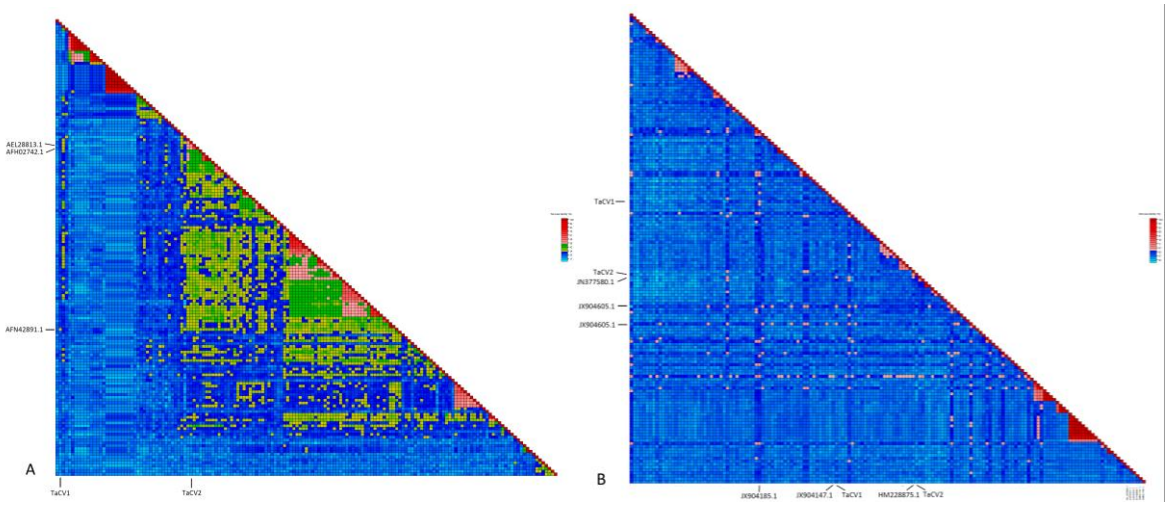


Figure 3: Colour coded pairwise identity matrices of Rep proteins (A) and complete genomes (B), showing the percentage of Rep amino acid and genome nucleotide identity of TaCV1 and TaCV2 with 159 CRESS DNA viruses.

The Bayesian analyses of the Rep proteins reveals that the Rep protein of TaCV1 forms a sister clade with a wide diversity of CRESS DNA Rep proteins, in which the Rep protein of TaCV2 is embedded (Fig. 4). The Rep protein of TaCV2 is most related to Rep proteins of bat circoviruses (AFH02742, AEL28813) (Fig. 4). The clade in which both viruses are placed infect hosts of wide phylogenetic origins, including insects and mammals (Fig. 4). Moreover, a considerable part of viruses in this clade were found in marine environments (Fig. 4). These findings suggest that extensive host switching has taken place in the evolutionary history of these viruses between hosts and habitats of with very diverse origins.

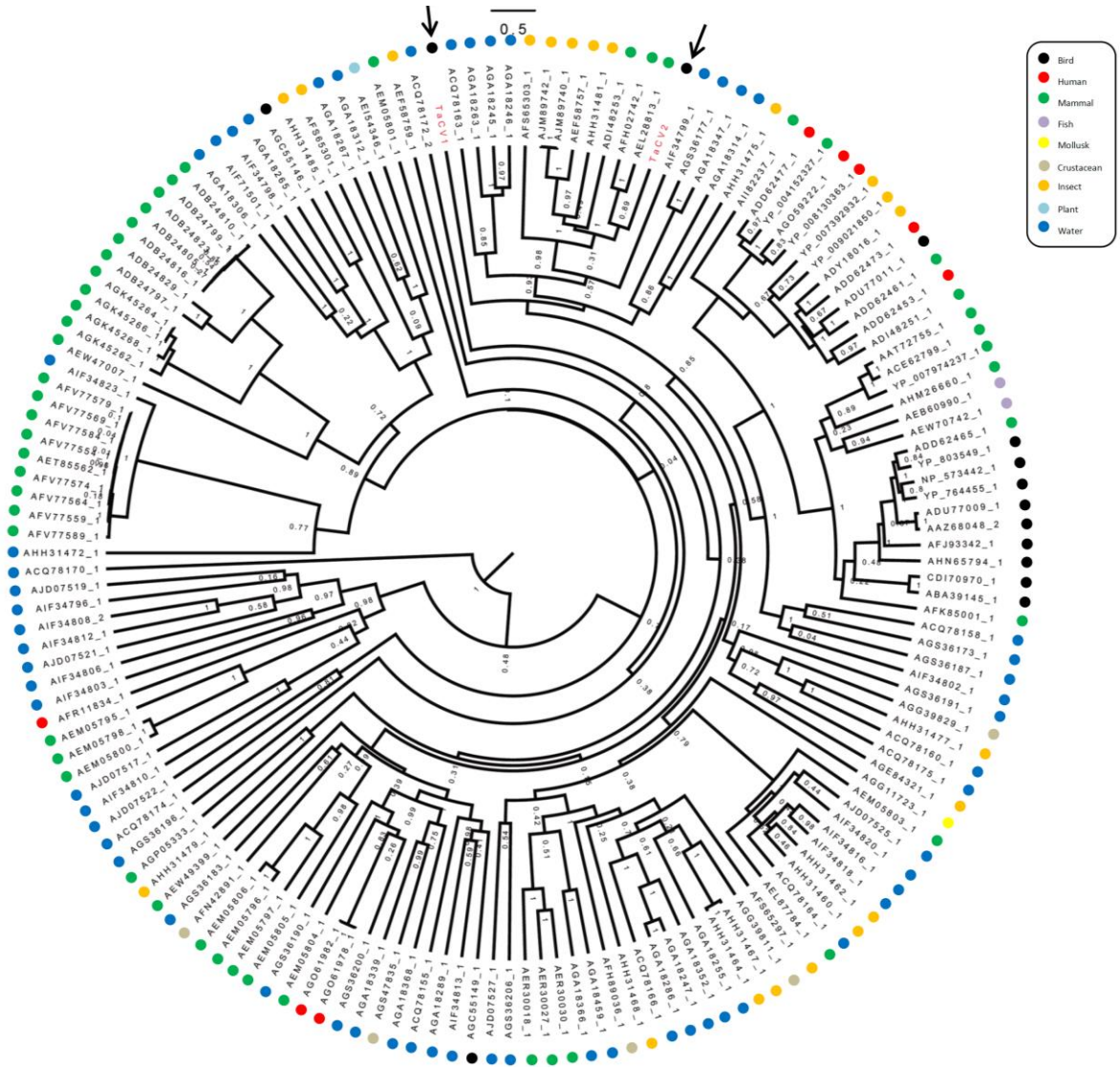


Figure 4: Evolutionary relationships of Rep proteins of the two genomes characterized in this study (TaCV1 and TaCV2; marked with arrows) inferred with Bayesian analyses. The colors at the end of the branches represent the host group or habitat from which the virus was isolated, showing extensive host switches have governed the evolution of CRESS DNA viruses. Numbers along the branches represent branch support (posterior probabilities).

ORF1 of TaCV1 and both ORFs of TaCV2 did not show any significant BLASTP or BLASTX matches on GenBank but the *in silico* analysis suggests that two of them could function as capsid proteins. ORF1 (TaCV1) and ORF2 (TaCV2) encoded two basic proteins of 24 and 20 KDa with and isoelectric point (pI) of 9.66 and 9.54 respectively, as opposed to ORF1/TaCV2 with pI 5. Basic amino acids (K+R) represented 20% of the total amino acids in both proteins but were not concentrated exclusively in the N-terminal region such as in other Cap proteins of circoviruses which normally contain several arginine-clusters. This highly basic region seems to be involved in packing of the viral genome into the viral capsid (Johne *et al.* 2004). Besides it is known that Cap protein participates in the attachment, entry and shuttling of the viral genome across the nuclear pore complex because for these viruses the host cell nucleus is the site of virus replication (Trible & Rowland 2012).

Moreover, a putative bipartite nuclear location signal (NLS) corresponds to the classic consensus sequence [$(K/R)_2X_{10\text{-}12}(K/R)_3$] which was located in the N-terminal region of ORF1 in TaCV1 (3-**KR**HTRSYLQEINTF**KKK**-19). This motif was first described in *Xenopus laevis* nucleoplasmine and has been found in varicella zoster virus (Huang *et al.* 2014). Overlapping with this region a DNA binding domain (2-SKRHTRSY-9) was identified in the same region. In contrast, no canonical NLS was found in ORF2 (TaCV2), although there was a potential motif (9-**RR**Y**KK**-13), slightly different from a type of monopartite NLS containing 3-5 basic amino acids with the weak consensus KR/KXR/K, similar to simian virus 40 large antigen (Chelsky, Ralph & Jonak 1989). Additionally the same motif was identified as a DNA binding region. The Rep protein of TaCV2 contained motifs related to rolling circle replication (24-FTLNN-28, 56-TPHLQG-61) or deoxynucleoside triphosphate (dNTP) binding sites (172-GxGKS-176). Other highly conserved motifs in *Circovirus* are identified and are slightly modified (196-WWNGY-200; 226-DRYP-229) (Li *et al.* 2010). The Rep protein of TaCV1 contained other different motifs (16-FTIFN-20; 54-PHIQG-58; 190- GxGKS-194).

Based on the pairwise energy profiles created by IUPred, ORF1 of TaCV1 and ORF2 from TaCV2 seem to be intrinsically unstructered over their entire length with the exception of a region between residues 10 and 50 (Fig. 5). Both ORFs show a very similar pairwise energy profile (Fig. 5). These residues also show α-helical structure, possibly relevant to the physiological structure of this protein. Within these residues ANCHOR detected putative binding regions when interacting with other globular proteins (Fig. 5). These represent sites that can undergo a disorder-to-order transition upon binding (Mészáros *et al.* 2009). We did not detect IDRs in ORF1 of TaCV2 with IUPred but futher analyses with PONDR-fit and DISPROT VL3 detected an IDR between residues 100 and 150 (Fig. 5). In summary, we were able to find a charachteristic IDR profile for ORF1 in TaCV1 and ORF2 in TaCV2 with a peak in predicted disorder from residue 50 onwards, which were different to the profiles presented in Rosario et al. (2015). On the other hand ORF1 of TaCV2 shows a peak approximately between residues 110 and 140 (PONDR-fit and DISPROT VL3), which also represents a new IDR profile for CRESS DNA viruses. Moreover, at least four regions can be identified as potential binding regions in ORF1 (TaCV1) and ORF2 (TaCV2) while none is recognized in ORF1 (TaCV2) by the ANCHOR-software (Fig. 5).

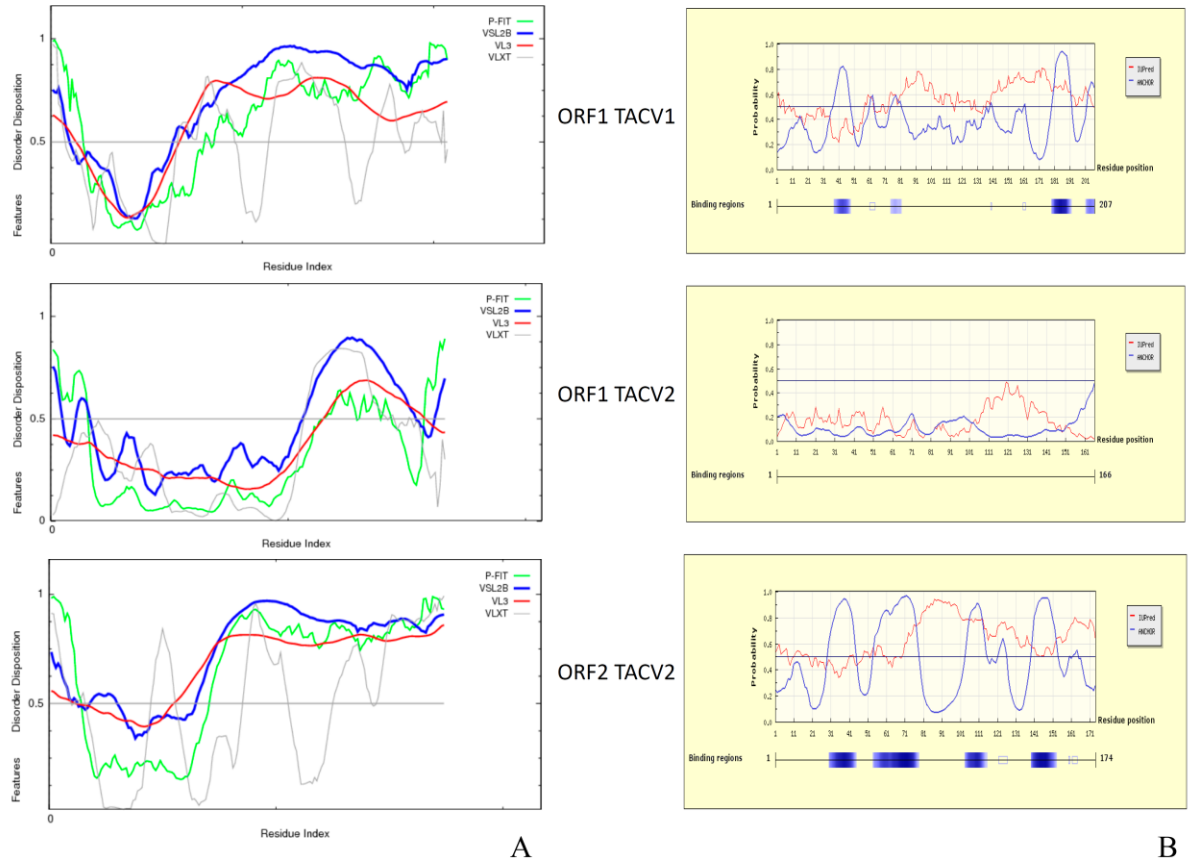


Figure 5: Representation of intrinsically disordered regions profiles of all putative ORFs of both CRESS DNA viruses described in this study. Putative binding regions within amino acid sequences are shown. Values above the 0.5 threshold indicate disordered regions and binding regions.

In summary, we describe two novel CRESS DNA genomes isolated from a lesion caused by *Avipoxvirus*, which add up to the phylogenetic and functional diversity of these viruses. Experiments are needed to understand the potential impacts of these viruses on avian health. Virtually nothing is known about CRESS virus prevalence in wild avian communities and this warrants further research. Since both new viruses were detected in a common tanager species, future studies on the prevalence of CRESS DNA viruses in the spectacular diversity of tanagers are warranted. We found new structural properties in the newly described CRESS DNA viruses, which arise as distinct from the types described in Rosario et al. (2015). These authors found two distinct IDR profiles which were concentrated within the first 50 amino acids towards the N-terminal. In contrast, we report two IDR profiles with intrinsic disorder from residue 50 towards the C-terminal. ORF1 of TaCV1 and ORF2 of TaCV2 present very similar profiles and reveal the existence of a putative Cap protein in both genomes. It has been shown that smaller viruses exhibit more intrinsic disorder which may be involved in encoding multifunctional proteins (Xue, Dunker & Uversky 2012; Pushker *et al.* 2013). Therefore we encourage analyzing IDR profiles of putative Cap proteins of already described and to-be-discovered CRESS DNA viruses, in order to uncover the incidence of different types of sequence rearrangements. The latter may prove very informative in terms of both functioning and classification of these viruses if they are usable as molecular signatures of phylogenetic origin. Along with the

characteristic IDR profile, motifs associated with rolling circle replication and NLS, we provide support for the existence of putative Cap proteins in CRESS DNA viruses. This approach may reveal new potential to understand the structural properties of this widespread and megadiverse group of viruses. Finally, we show evidence that extensive host switching between phylogenetically distant hosts has governed the evolutionary history of CRESS DNA viruses, which make these viruses good models to study the evolution of host specificity. This is especially important to understand their potential of colonizing new habitats and to monitor possible disease outbreaks.

Supplementary material

Chapter 1: Discovering potential sources of emerging pathogens: South America is a reservoir of generalist avian blood parasites.

Supplementary Table S1

The *Plasmodium* and *Haemoproteus* lineages found in each host species sampled in Wisui (Ecuador), and their local host range index (S^*_{TD} index). For every host species, the number of captured individuals (Cap), infected individuals (Inf) and parasite prevalence (Prev) were calculated[a]. Genbank accession numbers are shown.

Plasmodium

Lineage	Host	Cap	Inf	Prev	S^*_{TD}	Genbank Nr.
LEPCOR02	*Lepidothrix coronata*	35	3	0.09	4.664	KT373867
	Xenopipo holochlora	6	1	0.17		
	Myiobius atricaudus	1	1	1		
THAMAE01	*Thamnophilus aethiops*	4	1	0.25	3	KT373878
	Willisornis poecilinotus	12	1	0.08		
THACAE01	*Thamnomanes caesius*	8	1	0.13	3	KT373877
	Hylophylax naevius	12	1	0.08		
MYRMYO01	*Myrmoborus myotherinus*	14	1	0.07	6	KT373866
	Hylophylax naevius	12	1	0.08		
	Myrmeciza fortis	4	1	0.25		
	Gymnopithys leucaspis	13	1	0.08		
	Thamnomanes ardesiacus	6	1	0.17		
HYLSUB01	*Hyloctistes subulatus*	4	1	0.25	3	KT373872
	Syndactyla ruficollis	2	1	0.5		
LEPCOR01	*Lepidothrix coronata*	35	2	0.06	0	KT373871
MYRMAX01	*Myrmotherula axillaris*	5	1	0.2	3	KT373868
	Gymnopithys leucaspis	13	1	0.08		
LEPCOR04	*Lepidothrix coronata*	35	3	0.09	0	KT373874
MYRMYO02	*Myrmoborus myotherinus*	14	1	0.07	0	KT373875
PHAFUL01	*Phaeothlypis fulvicauda*	2	1	0.5	0	KT373876
HYPCAN01	*Hypocnemis cantator*	6	1	0.17	0	KT373873
TACRUB01	*Pipra erythrocephala*	6	1	0.17	0	KT373870
GYMLEU01	*Gymnopithys leucaspis*	13	1	0.08	0	KT373869

Haemoproteus

Lineage	Host	Cap	Inf	Prev	S^*_{TD}	Genbank Nr.
LEPCOR03	*Lepidothrix coronata*	35	3	0.09	8.476	KT373858
	Gymnopithys leucaspis	13	1	0.08		
	Hypocnemis cantator	6	1	0.17		
	Chiroxiphia pareola	12	3	0.25		
	Epinecrophylla erythrura	3	1	0.33		
	Pithys albifrons	10	1	0.1		
	Thamnomanes caesius	8	1	0.13		
GEOTRY01	*Geotrygon montana*	5	2	0.4	0	KT373865
MALFUS01	*Malacoptila fusca*	2	1	0.5	0	KT373864
HELSCH01	*Heliodoxa schreibersii*	1	1	1	0	KT373863
XENHOL01	*Xenopipo holochlora*	6	1	0.17	0	KT373862
PACPEC02	*Euphonia xanthogaster*	11	1	0.09	4	KT373859
	Saltator maximus	2	1	0.5		
EUXAN01	*Euphonia xanthogaster*	11	1	0.09	2	KT373860
	Euphonia laniirostris	1	1	1		
TANSCH01	*Tangara schrankii*	5	5	1	3	KT373861
	Tangara gyrola	1	1	1		
	Tangara chilensis	1	1	1		

[a]Other birds sampled that tested negative for parasites (number examined in parentheses): BUCCONIDAE: *Nonnula Brunnea* (2). CARDINALIDAE: Cyanocompsa cyanoides (3); *Saltator grossus* (1). COTINGIDAE: *Pipreola chlorolepidota* (2). DENDROCOLAPTIDAE: *Dendrocincla fuliginosa* (1); *Glyphorynchus spirirus* (21); *Xiphorhynchus ocellatus* (5). EMBERIZIDAE: *Arremon aurantiirostris* (1). FALCONIDAE: *Micrastur gilvicollis* (1). FORMICARIDAE: Formicarius analis (2); Myrmothera campanisona (2). FURNARIIDAE: *Automolus infuscatus* (3); *Automolus melanopezus* (1); *Automolus ochrolaemus* (3); *Phylidor ruficaudatum* (1); *Phylidor erythrocercum*

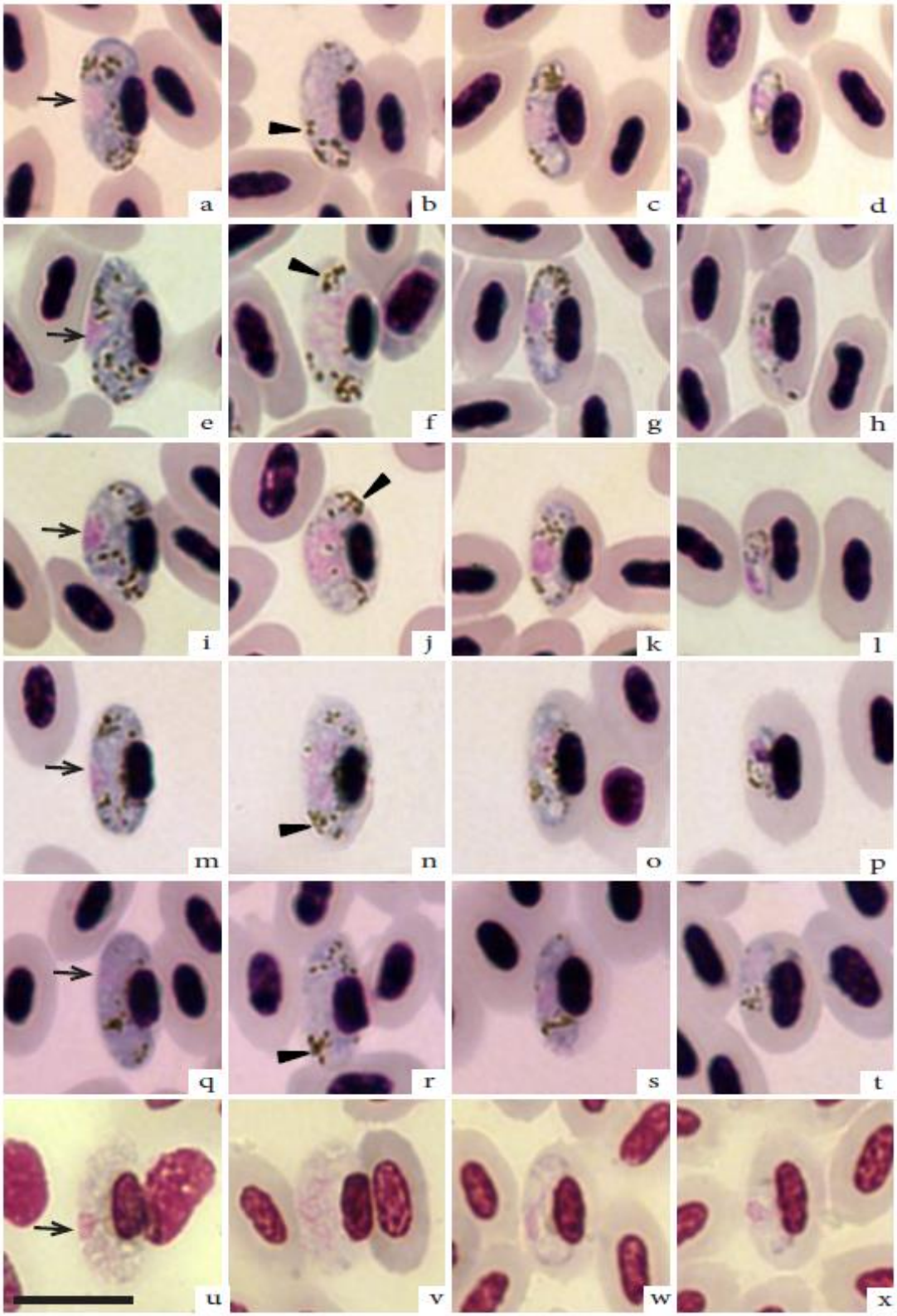
(1); *Sclerurus caudacutus* (1); *Sclerurus mexicanus* (1); *Xenops minutus* (4). PICIDAE: *Picumnus rufiventris* (5). PIPRIDAE: *Pipra pipra* (2); *Lepidothrix isodorei* (1); *Machaeropterus regulus* (3); *Manacus manacus* (4); *Xenopipo unicolor* (1). POLIOPTILIDAE: *Microbates cenereiventris* (3). THAMNOPHILIDAE: *Cercomacra serva* (2); *Dichrozona cincta* (1); *Frederickena unduliger* (1); *Myrmeciza melanoceps* (1); *Myrmotherula erythrina* (2); Myrmotherula menetriesii (2); *Epinecrophylla spodionota* (1); *Schistocichla leucostigma* (3); *Schistocichla schistacea* (1); *Thamnophilus schistaceus* (2). THRAUPIDAE: *Heterospingus xanthopygius* (1); *Lanio fulvus* (2). TROCHILIDAE: *Campylopterus largipennis* (1); *Eutoxeres aquila* (1); *Eutoxeres condamini* (3); *Phaethornis malaris* (5); *Thalurania furcata* (1); *Threnetes niger* (2). TROGLODYTIDAE: *Cyphorhinus arada* (3); *Henicorhina leucosticta* (3); *Microcerculus bambla* (1); *Microcerculus marginatus* (3); *Pheugopedius coraya* (1). TURDIDAE: *Catharus ustulatus* (2); *Turdus albicollis* (2). TYRANNIDAE: *Mionectes oleagineus* (9); *Mionectes olivaceus* (6); *Myiophobus cryptoxanthus* (1); *Myiotriccus ornatus* (2); *Platyrinchus coronatus* (2); *Poecilotriccus capitalis* (2); *Schiffornis turdina* (1); *Tolmomyias poliocephalus* (1). VIREONIDAE: *Hylophilus ochraeiceps* (3).

Chapter 2: Parasite specialization in a unique habitat: hummingbirds as reservoirs of generalist blood parasites of Andean birds.

Appendix S1: List of all captured bird species with numbers in parentheses. **Accipitridae (3)***; Accipiter striatus (3);* **Caprimulgidae (5)***; Nyctidromus albicollis (1); Systellura longirostris (2); Uropsalis segmentata (2);* **Cardinalidae (4)***; Pheucticus chrysogaster (3); Saltator cinctus (1);* **Columbidae (3)***; Leptotila pallida (3);* **Cotingidae (14)***; Pipreola riefferii (14);* **Cuculidae (2)***; Crotophaga sulcirostris (2);* **Emberizidae (78)***; Atlapetes latinuchus (21); Atlapetes pallidinucha (7); Arremon brunneinucha (2); Arremon torquatus (14); Catamenia homochroa (1); Catamenia inornata (1); Haplospiza rustica (7); Tiaris obscurus (19); Volatinia jacarina (5); Zonotrichia capensis (1);* **Fringillidae (3)***; Spinus psaltria (3);* **Furnariidae (74)***; Asthenes fuliginosa (1); Asthenes griseomurina (2); Cranioleuca antisiensis (2); Cranioleuca curtata (1); Dendrocincla tyrannina (1); Furnarius leucopus (9); Hellmayrea gularis (9); Lepidocolaptes lacrymiger (9); Margarornis squamiger (10); Synallaxis azarae (30);* **Grallariidae (5)***; Grallaria ruficapilla (2); Grallaria rufula (1); Grallaria squamigera (1); Grallaricula nana (1);* **Hirundinidae (1)***; Orochelidon flavipes (1);* **Icteridae (5)***; Amblycercus holosericeus (3); Dives warszewiczi (1); Icterus mesomelas (1);* **Parulidae (104)***; Basileuterus trifasciatus (16); Myioborus melanocephalus (7); Myioborus miniatus (9); Myiothlypis coronata (41); Myiothlypis fraseri (13); Myiothlypis luteoviridis (4); Myiothlypis nigrocristata (14);* **Picidae (3)***; Colaptes rivolii (2); Colaptes rubiginosus (1);* **Rhinocryptidae (10)***; Scytalopus latrans (1); Scytalopus robbinsi (1); Scytalopus sp. (8);* **Strigidae (3)***; Glaucidium peruanum (3);* **Thraupidae (109)***; Anisognathus igniventris (2); Anisognathus lacrymosus (10); Catamblyrhynchus diadema (10); Chlorornis riefferii (1); Chlorospingus canigularis (1); Cnemathraupis eximia (1); Diglossa albilatera (25); Diglossa caerulescens (4); Diglosssa cyanea (20); Diglossa lafresnayii (8); Dubusia taeniata (1); Hemispingus atropileus (3); Hemispingus verticalis (4); Iridosornis rufivertex (3); Pipraeidea melanonota (2); Tangara vassorii (2); Thlypopis sp. (1); Thlypopsis ornata (2); Thraupis cyanocephala (5); Thraupis episcopus (4);* **Tityridae (6)***; Pachyramphus albogriseus (3); Pachyramphus versicolor (1); Pachyramphus homochrous (2);* **Trochilidae (169)***; Adelomyia melanogenys (31); Amazilia alticola (17); Boissoneaua matthewsii (5); Chalcostigma herrani (4); Chalcostigma ruficeps (1); Coeligena iris (27); Coeligena lutetiae (1); Coeligena torquata (8); Colibri thalassinus (4); Doryfera ludovicae (1); Eriocnemis vestita (21); Heliangelus micraster (5); Heliangelus viola (13); Heliodoxa gularis (5); Heliodoxa schreibersii (1); Lafresnaya lafresnayi (6);*

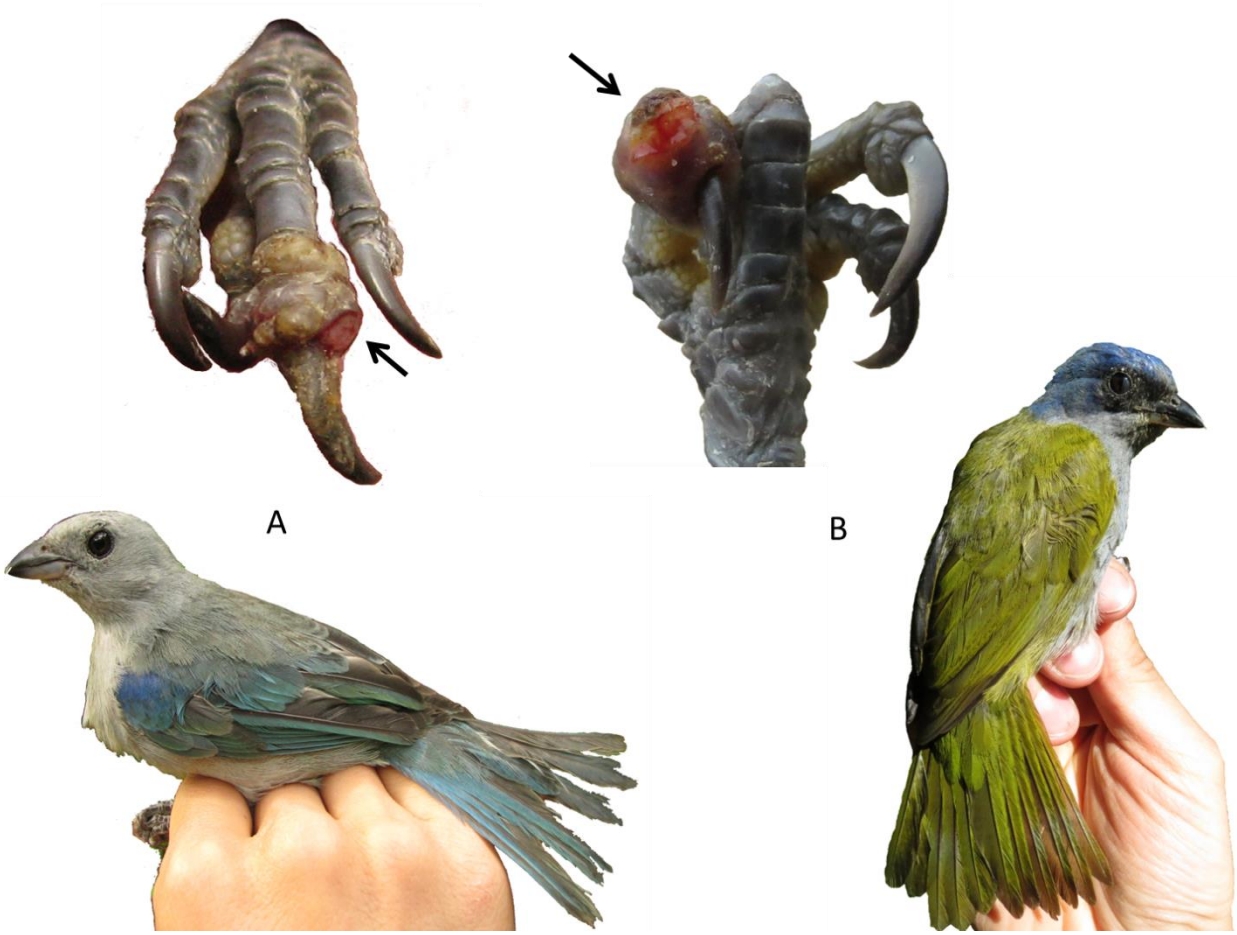
Lesbia nuna (1); Metallura tyrianthina (7); Phaethornis griseoregularis (11); **Troglodytidae (27)***; Campylorhynchus fasciatus (3); Cinnycerthia unirufa (15); Cistothorus platensis (1); Pheugopedius europhrys (1); Troglodytes aedon (2); Troglodytes solstitialis (5);* **Trogonidae (3)***; Trogon personatus (3);* **Turdidae (98)***; Catharus fuscater (8); Myadestes ralloides (54); Turdus fuscater (4); Turdus maculirostris (6); Turdus nigriceps (12); Turdus reevei (3); Turdus serranus (11);* **Tyrannidae (168)***; Elaenia albiceps (30); Elaenia pallatangae (3); Empidonax virescens (4); Euscarthmus meloryphus (1); Mecocerculus leucophrys (3); Mecocerculus stictopterus (4); Megarynchus pitangua (3); Mionectes striaticollis (81); Myiarchus tuberculifer (1); Myiophobus fasciatus (2); Nephelomyias lintoni (2); Ochthoeca cinnamomeiventris (2); Ochthoeca diadema (6); Ochthoeca frontalis (2); Ochthoeca rufipectoralis (9); Phyllomyias griseiceps (1); Phyllomyias nigrocapillus (1); Pseudotriccus ruficeps (3); Pyrrhomyias cinnamomeus (2); Sayornis nigricans (4); Tyrannus melancholicus (4);* **Vireonidae (8)***; Cyclarhis gujanensis (6); Vireo leucophrys (2).*

Figure S1: Circulating gametocytes of *Haemoproteus witti* in hummingbirds. *Haemoproteus witti* gametocytes in 6 different hummingbird species, including the type host: *Adelomyia melanogenys* (a-d), *Coeligena iris* (e-h), *Colibri thalassinus* (i-l), *Lafresnaya lafresnayi* (m-p), *Metallura tyrianthina* (q-t) and *Trochilus polytmus* (u-x). The first, second, third and fourth column show macrogametocytes, microgametocytes, medium-grown gametocytes and young gametocytes respectively. The type material (u-x) is slowly fading. Arrows indicate the pink parasite nucleus and arrowheads show pigment granules. Scale bar is 10 μm.


a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
v
w
x

Chapter 3: The Biological Background of a Recurrently Emerging Infectious Disease: Prevalence, Diversity and Host Specificity of Avipoxvirus in Wild Neotropical Birds

S1 Fig. Detail of *Avipoxvirus* lesions caused by the two new strains found in this study, from the blue-gray tanager *Thraupis episcopus* (A) and the blue-capped tanager *Thraupis cyanocephala* (B). Arrows indicate the lesions.



S1 Appendix. Screened bird families and species in Ecuador with number of captured individuals in parentheses.

Accipitridae (5): *Accipiter striatus (5),* **Caprimulgidae (5)**: *Systellura longirostris (2), Nyctiodromus albicollis (1), Uropsalis segmentata (2),* **Cardinalidae (4):** *Pheucticus chrysogaster (3), Saltator cinctus (1),* **Columbidae (3):** *Leptotila pallida (3),* **Cotingidae (14):** *Pipreola riefferii (14),* **Cuculidae (2):** *Crotophaga sulcirostris (2),* **Emberizidae (80):** *Atlapetes latinuchus (22), Atlapetes pallidinucha (7), Arremon brunneinucha (2), Arremon torquatus (14), Catamenia homochroa (1), Catamenia inornata (1), Haplospiza rustica (7), Tiaris obscurus (20), Volatinia jacarina (5), Zonotrichia capensis (1),* **Fringillidae (3):** *Carduelis psaltria (3),* **Furnariidae (76):** *Asthenes*

fuliginosa (1), Asthenes griseomurina (2), Cranioleuca antisiensis (2), Cranioleuca curtata (1), Dendrocincla tyrannina (1), Furnarius leucopus (9), Hellmayrea gularis (10), Lepidocolaptes lacrymiger (9), Margarornis squamiger (10), Synallaxis azarae (31), **Grallariidae (6):** *Grallaria ruficapilla (3), Grallaria rufula (1), Grallaria squamigera (1), Grallaricula nana (1),* **Hirundinidae (1):** *Orochelidon flavipes (1),* **Icteridae (5):** *Amblycercus holosericeus (3), Dives warszewiczi (1), Icterus mesomelas (1),* **Parulidae (116):** *Basileuterus trifasciatus (17), Myioborus melanocephalus (7), Myioborus miniatus (9), Myiothlypis coronata (49), Myiothlypis fraseri (14), Myiothlypis luteoviridis (4), Myiothlypis nigrocristata (15),* **Picidae (3):** *Colaptes rivolii (2), Colaptes rubiginosus (1),* **Rhinocryptidae (10):** *Scytalopus latrans (1), Scytalopus robbinsi (1), Scytalopus sp. (8),* **Strigidae (3):** *Glaucidium peruanum (3),* **Thraupidae (112):** *Anisognathus igniventris (2), Anisognathus lacrymosus (10), Catamblyrhynchus diadema (10), Chlorornis riefferii (1), Chlorospingus canigularis (1), Diglossa albilatera (27), Cnemathraupis eximia (1), Diglossa lafresnayii (8), Diglossa caerulescens (4), Diglossa cyanea (21), Dubusia taeniata (1), Hemispingus atropileus (3), Hemispingus verticalis (4), Iridosornis rufivertex (3), Pipraeidea melanonota (2), Tangara vassorii (2), Thlypopis sp. (1), Thlypopsis ornata (2), Thraupis cyanocephala (5), Thraupis episcopus (4),* **Tityridae (6):** *Pachyramphus albogriseus (3), Pachyramphus versicolor (1), Pachyramphus homochrous (2),* **Trochilidae (172):** *Adelomyia melanogenys (32), Amazilia alticola (17), Boissoneaua matthewsii (5), Chalcostigma herrani (4), Chalcostigma ruficeps (1), Coeligena iris (28), Coeligena lutetiae (1), Coeligena torquata (8), Colibri thalassinus (4), Doryfera ludovicae (1), Eriocnemis vestita (21), Heliangelus micraster (5), Heliangelus viola (14), Heliodoxa gularis (5), Heliodoxa schreibersii (1), Lafresnaya lafresnayi (7), Lesbia nuna (1), Metallura tyrianthina (7), Phaethornis griseoregularis (11),* **Troglodytidae (28):** *Campylorhynchus fasciatus (3), Cinnycerthia unirufa (15), Cistothorus platensis (1), Pheugopedius europhrys (1), Troglodytes aedon (3), Troglodytes solstitialis (5), Trogonidae (3), Trogon personatus (3),* **Turdidae (99):** *Catharus fuscater (8), Myadestes ralloides (55), Turdus fuscater (4), Turdus maculirostris (6), Turdus nigriceps (12), Turdus reevei (3), Turdus serranus (11),* **Tyrannidae (177):** *Elaenia albiceps (30), Elaenia palletangae (3), Empidonax virescens (4), Euscarthmus meloryphus (1), Mecocerculus leucophrys (3), Mecocerculus stictopterus (4), Megarynchus pitangua (3), Mionectes striaticollis (89), Myiarchus tuberculifer (1), Myiophobus fasciatus (2), Nephelomyias lintoni (2), Ochthoeca cinnamomeiventris (2), Ochthoeca diadema (6), Ochthoeca frontalis (2), Ochthoeca rufipectoralis (10), Phyllomyias griseiceps (1), Phyllomyias nigrocapillus (1), Pseudotriccus ruficeps (3), Pyrrhomyias cinnamomea (2), Sayornis nigricans (4), Tyrannus melancholicus (4),* **Vireonidae (8):** *Cyclarhis gujanensis (6), Vireo leucophrys (2),* Unknown spp. (2).

Chapter 4: Characterization and evolutionary relationships of two novel CRESS DNA viruses isolated from an *Avipoxvirus* lesion of a common bird in Ecuador.

Table A.1: List of primer sets used for PCR and walking sequencing

Virus	Primer	Type	Sequence	Used for
TaCV1	CIR1000F1S	PCR Back-to-back	5'-GAAAGAAACGTGATGACTCC-3'	RCA/Not amplified sample
TaCV1	CIR1000R1S	PCR Back-to-back	5'-TCGAATTTGGAGTCATCACG-3'	RCA/Not amplified sample
TaCV1	CIR1000F2	PCR Non-overlapping	5'-CAAGCGGTAGTGGTAAAAG-3'	RCA/Not amplified sample
TaCV1	CIR1000R2	PCR Non-overlapping	5'-CAAGCTGGAGACCGAAACT-3'	RCA/Not amplified sample
TaCV1	Mi500F1	Sequencing	5'-AGCGAGCATTCCCATTTTTCA-3'	Cloned sequences/RCA
TaCV1	Mi10R1	Sequencing	5'-GTTTTCTGGTCCAAGCGGTAGTG-3'	Cloned sequences/RCA
TaCV1	Mi500F2	Sequencing	5'-TAAAACGGAGTGTAAGTGGT-3'	Cloned sequences/RCA
TaCV1	Mi10R2	Sequencing	5'-AAGGCGAGTAATAACAGTAAGTCT-3'	Cloned sequences/RCA
TaCV1	Mi10F3	Sequencing	5'-CATTACCACTTACACTCCGTTTTA-3'	Cloned sequences
TaCV1	Mi500R3	Sequencing	5'-TGTTATTACTCGCCTTCTCAG-3'	Cloned sequences
TaCV1	Mi1000/walk/FW1	Sequencing	5'-AACTCCTTCGTGATATAAATGTAAT-3'	RCA
TaCV1	Mi1000/walk/RV1	Sequencing	5'-ACGAGGAAGGGTTTTGTAATAATA-3'	RCA
TaCV1	Mi1000/walk/FW2	Sequencing	5'-TTCTCTTTTAACTCCTTCGTGATA-3'	RCA
TaCV1	Mi1000/walk/RV2	Sequencing	5'-CGATATTTAACGTTGGTGAGG-3'	RCA
TaCV1	Mi1000/walk/FW3	Sequencing	5'-ATCATTTGGTTATATCATTTTA-3'	RCA
TaCV1	Mi1000/walk/RV3	Sequencing	5'-TCGATAGAAACAAGATACCC-3'	RCA
TaCV1	M13RP	Sequencing	5'-CAGGAAACAGCTATGACC-3'	Cloned sequences
TaCV1	M13FP	Sequencing	5'-TGTAAAACGACGGCCAGT-3'	Cloned sequences
TaCV2	CIR3000F1S	PCR Back-to-back	5'-TAATAGAAACAAAAATAAAGAACC-3'	RCA/Not amplified sample
TaCV2	CIR3000R1S	PCR Back-to-back	5'-TTGTTTCTATTATGTTGTTTATTG-3'	RCA/Not amplified sample
TaCV2	CIR3000F2	PCR Non-overlapping	5'-GCTTATGAAATCTTGCACTACTTG-3'	RCA/Not amplified sample
TaCV2	CIR3000R2	PCR Non-overlapping	5'-TATGTGAATGTAGAAAGTGAGTTGG-3'	RCA/Not amplified sample
TaCV2	Mi3000/walk/FW1	Sequencing	5'-TCAAAAATAGTGGAAAAATAGG-3'	RCA
TaCV2	Mi3000/walk/RV1	Sequencing	5'-CCTCTTCTTTCTTCCCAATGTA-3'	RCA
TaCV2	Mi3000/walk/FW2	Sequencing	5'-TCTAAAGCTTCATCACTCAATCTAT-3'	RCA
TaCV2	Mi3000/walk/RV2	Sequencing	5'-TAGTCCATGGCATATTCCTTTTTA-3'	RCA
TaCV2	Mi3000/walk/FW3	Sequencing	5'-TTGGAAAGATGACACTAAATGGT-3'	RCA
TaCV2	Mi3000/walk/RV3	Sequencing	5'-CTTTCTGTTATAGTAGGGATTTTC-3'	RCA

References

Agosta, S.J. & Klemens, J.A. (2008) Ecological fitting by phenotypically flexible genotypes: implications for species associations, community assembly and evolution. *Ecology Letters*, **11**, 1123–1134.

Ahumada, J.A., Lapointe, D.A. & Samuel, M.D. (2004) Modeling the population dynamics of *Culex quinquefasciatus* (Diptera: Culicidae), along an elevational gradient in Hawaii. *Journal of Medical Entomology*, **41**, 1157–1170.

Araujo, S.B.L., Braga, M.P., Brooks, D.R., Agosta, S.J., Hoberg, E.P., Von Hartenthal, F.W. & Boeger, W.A. (2015) Understanding host-switching by ecological fitting. *PLoS ONE*, **10**, 1–17.

Aruch, S., Atkinson, C.T., Savage, A.F. & Lapointe, D.A. (2007) Prevalence and distribution of pox-like lesions, avian malaria, and mosquito vectors in Kipahulu Valley, Haleakala National Park, Hawai'i, USA. *Journal of wildlife diseases*, **43**, 567–575.

Asghar, M., Hasselquist, D., Zehtindjiev, P., Westerdahl, H. & Bensch, S. (2015) Hidden costs of infection: Chronic malaria accelerates telomere degradation and senescence in wild birds. *Science*, **347**, 436–438.

Atkinson, C.T. & Lapointe, D.A. (2009) Introduced avian diseases, climate change, and the future of Hawaiian honeycreepers. *Journal of Avian Medicine and Surgery*, **23**, 53–63.

Atkinson, C.T., Lease, J.K., Dusek, R.J. & Samuel, M.D. (2005) Prevalence of pox-like lesions and malaria in forest bird communities on Leeward Mauna Loa Volcano, Hawaii . *The Condor*, **107**, 537–546.

Beadell, J.S., Covas, R., Gebhard, C., Ishtiaq, F., Melo, M., Schmidt, B.K., Perkins, S.L., Graves, G.R. & Fleischer, R.C. (2009) Host associations and evolutionary relationships of avian blood parasites from West Africa. *International journal for parasitology*, **39**, 257–266.

Beadell, J.S., Gering, E., Austin, J., Dumbacher, J.P., Peirce, M.A., Pratt, T.K., Atkinson, C.T. & Fleischer, R.C. (2004) Prevalence and differential host-specificity of two avian blood parasite genera in the Australo-Papuan region. *Molecular Ecology*, **13**, 3829–3844.

Beck, E., Bendix, J., Kottke, I., Makeschin, F. & Mosandl, R. (2006) *Gradients in a Tropical Mountain Ecosystem of Ecuador*. Springer-Verlag, Berlin, Germany.

Belo, N.O., Pinheiro, R.T., Reis, E.S., Ricklefs, R.E. & Braga, É.M. (2011) Prevalence and lineage diversity of avian haemosporidians from three distinct cerrado habitats in Brazil. *PloS one*, **6**, e17654.

Bensch, S., Hellgren, O. & Peréz-Tris, J. (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome b lineages. *Molecular ecology resources*, **9**, 1353–1358.

Bensch, S., Pérez-Tris, J., Waldenström, J. & Hellgren, O. (2004) Linkage between nuclear and mitochondrial DNA sequences in avian malaria parasites: multiple cases of cryptic speciation? *Evolution*, **58**, 1617–1621.

Bensch, S., Stjernman, M., Hasselquist, D., Orjan, O., Hannson, B., Westerdahl, H. & Pinheiro, R.T. (2000) Host specificity in avian blood parasites: a study of Plasmodium and Haemoproteus mitochondrial DNA amplified from birds. *Proceedings of the Royal Society B: Biological Sciences*, **267**, 1583–1589.

Benson, G. (1999) Tandem repeats finder: a program to analyze DNA sequences. *Nucleic acids*

research, **27**, 573–580.

Bolte, A.L., Meurer, J. & Kaleta, E.F. (1999) Avian host spectrum of avipoxviruses. *Avian Pathology*, **28**, 415–432.

Bongers, F., Charles-Dominique, P., Forget, P.-M. & Théry, M. (2001) *Nouragues: Dynamics and Plant-Animal Interactions in a Neotropical Rainforest.* Dordrecht, Kluwer Academic Publishers.

Bonneaud, C., Mazuc, J., Gonzalez, G., Haussy, C., Chastel, O., Faivre, B. & Sorci, G. (2003) Assessing the cost of mounting an immune response. *The American Naturalist*, **161**, 367–379.

Bouckaert, R., Heled, J., Kühnert, D., Vaughan, T., Wu, C.-H., Xie, D., Suchard, M.A., Rambaut, A. & Drummond, A.J. (2014) BEAST 2: a software platform for bayesian evolutionary analysis. *PLoS Computational Biology*, **10**, e1003537.

Boyd, I.L., Freer-Smith, P.H., Gilligan, C.A. & Godfray, H.C.J. (2013) The consequence of tree pests and diseases for ecosystem services. *Science*, **342**, 1235773.

Brito, M. & Pozo-Zamora, G. (2013) Una nueva especie de rana terrestre del género *Pristimantis* (Amphibia: Craugastoridae), de la cordillera de Kutukú, Ecuador. *Papéis Avulsos de Zoologia*, **53**, 315–325.

Bull, J.J. & Lauring, A.S. (2014) Theory and empiricism in virulence evolution. *PLoS pathogens*, **10**, 1–3.

Cañadas Cruz, L. (1983) *El Mapa Bioclimático Y Ecológico Del Ecuador*. Quito: Banco central del Ecuador.

Care, Ministerio del Ambiente, Unión Europea & Tinker Foundation. (2012) *Plan de Manejo Actualizado Y Priorizado Del Bosque Protector Kutukú, Shaimi, 2012-2017.* Ministerio del Ambiente, Macas, Ecuador.

Carrete, M., Serrano, D., Illera, J.C., López, G., Vögeli, M., Delgado, A. & Tella, J.L. (2009) Goats, birds, and emergent diseases: apparent and hidden effects of exotic species in an island environment. *Ecological Applications*, **19**, 840–853.

Chelsky, D., Ralph, R. & Jonak, G. (1989) Sequence requirements for synthetic peptide-mediated translocation to the nucleus. *Molecular and cellular biology*, **9**, 2487–2492.

Civitello, D.J., Cohen, J., Fatima, H., Halstead, N.T., Liriano, J., McMahon, T.A., Ortega, C.N., Sauer, E.L., Sehgal, T., Young, S. & Rohr, J.R. (2015) Biodiversity inhibits parasites: broad evidence for the dilution effect. *Proceedings of the National Academy of Sciences*, **112**, 8667–8671.

Clark, N.J., Clegg, S.M. & Lima, M.R. (2014) A review of global diversity in avian haemosporidians (*Plasmodium* and *Haemoproteus*: Haemosporida): new insights from molecular data. *International Journal for Parasitology*, **44**, 329–338.

Clayton, D.H., Bush, S.E. & Johnson, K.P. (2004) Ecology of congruence: past meets present. *Systematic Biology*, **53**, 165–173.

Clayton, D.H. & Moore, J. (1997) *Host-Parasite Evolution: General Principles and Avian Models*. Oxford University Press, Oxford, UK.

Cleaveland, S., Laurenson, M.K. & Taylor, L.H. (2001) Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. *Proceedings of the Royal Society B: Biological Sciences*, **356**, 991–999.

Cokol, M., Nair, R. & Rost, B. (2000) Finding nuclear localization signals. *EMBO Reports*, **1**, 411–415.

Combes, C. (1997) Fitness of parasites: pathology and selection. *International Journal for Parasitology*, **27**, 1–10.

Combes, C. (2001) *Parasitism: The Ecology and Evolution of Intimate Interactions*. The University of Chicago Press.

Cornet, S., Nicot, A., Rivero, A. & Gandon, S. (2014) Evolution of plastic transmission strategies in avian malaria. *PLoS pathogens*, **10**, e1004308.

Cribari-Neto, F. & Zeileis, A. (2010) Beta regression in R. *Journal of Statistical Software*, **34**, 1–24.

Daszak, P., Cunningham, A.A. & Hyatt, D.A. (2000) Emerging infectious diseases of wildlife: threats to biodiversity and human health. *Science*, **287**, 443–449.

Davidson, W.R., Kellogg, F.E. & Doster, G.L. (1980) An epornitic of avian pox in wild bobwhite quail. *Journal of wildlife diseases*, **16**, 293–8.

Davies, R.G., Orme, C.D.L., Storch, D., Olson, V.A., Thomas, G.H., Ross, S.G., Ding, T.-S., Rasmussen, P.C., Bennett, P.M., Owens, I.P.., Blackburn, T.M. & Gaston, K.J. (2007) Topography, energy and the global distribution of bird species richness. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 1189–1197.

Dayaram, A., Goldstien, S., Argüello-Astorga, G.R., Zawar-Reza, P., Gomez, C., Harding, J.S. & Varsani, A. (2015) Diverse small circular DNA viruses circulating amongst estuarine molluscs. *Infection, Genetics and Evolution*, **31**, 284–295.

Dean, F.B., Nelson, J.R., Giesler, T.L. & Lasken, R.S. (2001) Rapid amplification of plasmid and phage DNA using Phi 29 DNA polymerase and multiply-primed rolling circle amplification. *Genome research*, **11**, 1095–9.

Delwart, E. & Li, L. (2012) Rapidly expanding genetic diversity and host range of the *Circoviridae* viral family and other Rep encoding small circular ssDNA genomes. *Virus Research*, **164**, 114–121.

Dobson, A. (2004) Population dynamics of pathogens with multiple host species. *The American naturalist*, **164**, 64–78.

Dosztanyi, Z., Csizmok, V., Tompa, P. & Simon, I. (2005) IUPred: web server for the prediction of intrinsically unstructured regions of proteins based on estimated energy content. *Bioinformatics*, **21**, 3433–3434.

Dosztányi, Z., Mészáros, B. & Simon, I. (2009) ANCHOR: web server for predicting protein binding regions in disordered proteins. *Bioinformatics*, **25**, 2745–2746.

Duffy, S., Shackelton, L.A. & Holmes, E.C. (2008) Rates of evolutionary change in viruses: patterns and determinants. *Nature Reviews Genetics*, **9**, 267–276.

Dunker, A.K., Lawson, J.D., Brown, C.J., Williams, R.M., Romero, P., Oh, J.S., Oldfield, C.J., Campen, A.M., Ratliff, C.M., Hipps, K.W., Ausio, J., Nissen, M.S., Reeves, R., Kang, C., Kissinger, C.R., Bailey, R.W., Griswold, M.D., Chiu, W., Garner, E.C. & Obradovic, Z. (2001) Intrinsically disordered protein. *Journal of molecular graphics and modelling*, **3263**, 26–59.

Dunlap, D.S., Ng, T.F.F., Rosario, K., Barbosa, J.G., Greco, A.M., Breitbart, M. & Hewson, I. (2013) Molecular and microscopic evidence of viruses in marine copepods. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 1375–1380.

Durrant, K.L., Beadell, J.S., Ishtiaq, F., Graves, G.R., Olson, S.L., Gering, E., Peirce, M. a., Milensky, C.M., Schmidt, B.K., Gebhard, C. & Fleischer, R.C. (2006) Avian hematozoa in South America: a comparison of temperate and tropical zones. *Ornitological Monographs*, **60**, 99–111.

Dyer, L.A., Singer, M.S., Lill, J.T., Stireman III, J.O., Gentry, G.L., Marquis, R.J., Ricklefs, R.E., Greeney, H.F., Wagner, D.L., Morais, H.C., Diniz, I.R., Kursar, T. a & Coley, P.D. (2007) Host specificity of Lepidoptera in tropical and temperate forests. *Nature*, **448**, 696–699.

Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acid Research*, **32**, 1792–1797.

Ewen, J.G., Bensch, S., Blackburn, T.M., Bonneaud, C., Brown, R., Cassey, P., Clarke, R.H. & Pérez-Tris, J. (2012) Establishment of exotic parasites: the origins and characteristics of an avian malaria community in an isolated island avifauna. *Ecology letters*, **15**, 1112–1118.

Fallon, S.M., Ricklefs, R.E., Swanson, B.L. & Bermingham, E. (2003) Detecting avian malaria: an improved polymerase chain reaction diagnostic. *The Journal of parasitology*, **89**, 1044–1047.

Fridolfsson, A.-K. & Ellegren, H. (1999) A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology*, **30**, 116–121.

Fuller, T., Bensch, S., Müller, I., Novembre, J., Pérez-Tris, J., Ricklefs, R.E., Smith, T.B. & Waldenström, J. (2012) The ecology of emerging infectious diseases in migratory birds: an assessment of the role of climate change and priorities for future research. *EcoHealth*, **9**, 80–88.

Futuyma, D.J. & Moreno, G. (1988) The evolution of ecological specialization. *Annual Review of Ecology and Systematics*, **19**, 207–233.

Gager, A.B., Del Rosario Loaiza, J., Dearborn, D.C. & Bermingham, E. (2008) Do mosquitoes filter the access of *Plasmodium* cytochrome b lineages to an avian host? *Molecular Ecology*, **17**, 2552–2561.

Galen, S.C. & Witt, C.C. (2014) Diverse avian malaria and other haemosporidian parasites in Andean house wrens: evidence for regional co-diversification by host-switching. *Journal of Avian Biology*, **45**, 374–386.

Gao, F., Bailes, E., Robertson, D.L., Chen, Y., Rodenburg, C.M., Michael, S.F., Cummins, L.B., Arthur, L.O., Peeters, M., Shaw, G.M., Sharp, P.M. & Hahn, B.H. (1999) Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. *Nature*, **397**, 436–441.

Garamszegi, L.Z. (2006) The evolution of virulence and host specialization in malaria parasites of primates. *Ecology letters*, **9**, 933–940.

Garigliany, M.-M., Borstler, J., Jost, H., Badusche, M., Desmecht, D., Schmidt-Chanasit, J. & Cadar, D. (2015) Characterization of a novel circo-like virus in *Aedes vexans* mosquitoes from Germany: evidence for a new genus within the family *Circoviridae*. *Journal of General Virology*, **96**, 915–920.

Giraudeau, M., Mousel, M., Earl, S. & McGraw, K. (2014) Parasites in the city: degree of urbanization predicts poxvirus and coccidian infections in house finches (*Haemorhous mexicanus*). *PLoS ONE*, **9**.

Glomski, C.A. & Pica, A. (2011) *The Avian Erythrocyte: Its Phylogenetic Odyssey*. CRC Press.

González, A.D., Lotta, I.A., García, L.F., Moncada, L.I. & Matta, N.E. (2015) Avian haemosporidians from Neotropical highlands: evidence from morphological and molecular data. *Parasitology International*, **64**, 48–59.

Graham, C.H., Parra, J.L., Rahbek, C. & McGuire, J.A.A. (2009) Phylogenetic structure in tropical hummingbird communities. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 19673–19678.

Green, M., Hughes, H., Sambrook, J. & MacCallum, P. (2012) *Molecular Cloning: A Laboratory Manual (Fourth Edition)*. CSH Press.

Gregory, R.D., Keymer, A.E. & Harvey, P.H. (1996) Helminth parasite richness among vertebrates. *Biodiversity and Conservation*, **5**, 985–997.

Gyuranecz, M., Foster, J.T., Dán, Á., Ip, H.S., Egstad, K.F., Parker, P.G., Higashiguchi, J.M., Skinner, M.A., Höfle, U., Kreizinger, Z., Dorrestein, G.M., Solt, S., Sós, E., Kim, Y.J., Uhart, M., Pereda, A., González-Hein, G., Hidalgo, H., Blanco, J.-M. & Erdélyi, K. (2013) Worldwide phylogenetic relationship of avian poxviruses. *Journal of virology*, **87**, 4938–4951.

Ha, H.J., Banda, M., Alley, M.R., Howe, L. & Gartrell, B.D. (2013) The seroprevalence of Avipoxvirus and its association with avian malaria (*Plasmodium* spp.) infection in introduced passerine birds in the southern regions of the North Island of New Zealand. *Avian diseases*, **57**, 109–115.

Hall, T. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Haller, S.L., Peng, C., McFadden, G. & Rothenburg, S. (2014) Poxviruses and the evolution of host range and virulence. *Infection, Genetics and Evolution*, **21**, 15–40.

Han, B.A., Schmidt, J.P., Bowden, S.E. & Drake, J.M. (2015) Rodent reservoirs of future zoonotic diseases. *Proceedings of the National Academy of Sciences*, **112**, 7039–7044.

Harrigan, R.J., Sedano, R., Chasar, A.C., Chaves, J.A., Nguyen, J.T., Whitaker, A. & Smith, T.B. (2014) New host and lineage diversity of avian haemosporidia in the northern Andes. *Evolutionary Applications*, 1–13.

He, B., Wang, K., Liu, Y., Xue, B., Uversky, V.N. & Dunker, A.K. (2009) Predicting intrinsic disorder in proteins: an overview. *Cell research*, **19**, 929–49.

Hellgren, O., Pérez-Tris, J. & Bensch, S. (2009) A jack-of-all-trades and still a master of some: prevalence and host range in avian malaria and related blood parasites. *Ecology*, **90**, 2840–2849.

Hellgren, O., Waldenström, J. & Bensch, S. (2004) A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. *The Journal of parasitology*, **90**, 797–802.

Hellgren, O., Waldenström, J., Peréz-Tris, J., Szöll, E., Si, Ö., Hasselquist, D., Križanauskienė, A., Ottosson, U., Bensch, S. & Si, O. (2007) Detecting shifts of transmission areas in avian blood parasites - a phylogenetic approach. *Molecular Ecology*, **16**, 1281–1290.

Hilty, S. & Brown, W. (1986) *A Guide to the Birds of Colombia*. Princeton University Press.

Hoberg, E.P. & Brooks, D.R. (2008) A macroevolutionary mosaic: episodic colonization and diversification in complex host-parasite systems. *Journal of Biogeography*, **35**, 1533–1550.

Huang, Y., Zhang, J., Halawa, M.A. & Yao, S. (2014) Nuclear localization signals of varicella zoster virus ORF4. *Virus Genes*, **48**, 243–251.

Illera, J.C., Emerson, B.C. & Richardson, D.S. (2008) Genetic characterization, distribution and prevalence of avian pox and avian malaria in the Berthelot's pipit (*Anthus berthelotii*) in Macaronesia. *Parasitology research*, **103**, 1435–1443.

Ishtiaq, F., Gering, E., Rappole, J.H., Rahmani, A.R., Jhala, Y. V, Dove, C.J., Milensky, C.M., Olson, S.L., Peirce, M. a. & Fleischer, R.C. (2007) Prevalence and diversity of avian hematozoan parasites in Asia: a regional survey. *Journal of wildlife diseases*, **43**, 382–398.

Jackson, B., Varsani, A., Holyoake, C., Jakob-hoff, R., Robertson, I., Mcinnes, K., Empson, R., Gray, R., Nakagawa, K. & Warren, K. (2015) Emerging infectious disease or evidence of endemicity? A multi-season study of beak and feather disease virus in wild red-crowned parakeets (*Cyanoramphus novaezelandiae*). *Archives of Virology*, **160**, 2283–2292.

Jarmin, S., Manvell, R., Gough, R.E., Laidlaw, S.M. & Skinner, M.A. (2006) *Avipoxvirus* phylogenetics: identification of a PCR length polymorphism that discriminates between the two major clades. *The Journal of general virology*, **87**, 2191–2201.

Jetz, W., Thomas, G.H.H., Joy, J.B.B., Hartmann, K. & Mooers, A. O.O. (2012) The global diversity of birds in space and time. *Nature*, **491**, 1–5.

Johne, R., Müller, H., Rector, A., van Ranst, M. & Stevens, H. (2009) Rolling-circle amplification of viral DNA genomes using phi29 polymerase. *Trends in microbiology*, **17**, 205–211.

Johne, R., Raue, R., Grund, C., Kaleta, E.F. & Müller, H. (2004) Recombinant expression of a truncated capsid protein of beak and feather disease virus and its application in serological tests. *Avian pathology*, **33**, 328–336.

Johnson, P.T.J., Preston, D.L., Hoverman, J.T. & Richgels, K.L.D. (2013a) Biodiversity decreases disease through predictable changes in host community competence. *Nature*, **494**, 230–233.

Johnson, P.T.J., Preston, D.L., J.T., H. & LaFonte, B.E. (2013b) Host and parasite diversity jointly control disease risk in complex communities. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 16916–16921.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. & Daszak, P. (2008) Global trends in emerging infectious diseases. *Nature*, **451**, 990–993.

Keesing, F., Belden, L.K., Daszak, P., Dobson, A., Harvell, C.D., Holt, R.D., Hudson, P., Jolles, A.E., Jones, K.E., Mitchell, C.E., Myers, S.S., Bogich, T. & Ostfeld, R.S. (2010) Impacts of biodiversity on the emergence and transmission of infectious diseases. *Nature*, **468**, 647–652.

Keesing, F., Holt, R.D. & Ostfeld, R.S. (2006) Effects of species diversity on disease risk. *Ecology Letters*, **9**, 485–98.

King, A.M.Q., Adams, M.J., Carstens, E.B. & Lefkowitz, E.J. (2012) *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses.* Elsevier Academic Press, San Diego, USA.

Kleindorfer, S. & Dudaniec, R.Y. (2006) Increasing prevalence of avian poxvirus in Darwin's finches and its effect on male pairing success effect on male pairing success. *Journal of Avian Biology*, **37**, 69–76.

Kraberger, S., Argüello-Astorga, G.R., Greenfield, L.G., Galilee, C., Law, D., Martin, D.P. & Varsani, A. (2015) Characterisation of a diverse range of circular replication-associated protein encoding DNA viruses recovered from a sewage treatment oxidation pond. *Infection, Genetics and Evolution*, **31**, 73–86.

Križanauskienė, A., Hellgren, O., Kosarev, V., Sokolov, L., Bensch, S. & Valkiūnas, G. (2006) Variation in host specificity between species of avian hemosporidian parasites: evidence from parasite morphology and cytochrome B gene sequences. *The Journal of parasitology*, **92**, 1319–1324.

Krupovic, M., Zhi, N., Li, J., Hu, G., Koonin, E. V., Wong, S., Shevchenko, S., Zhao, K. & Young, N.S. (2015) Multiple layers of chimerism in a single-stranded DNA Virus discovered by deep sequencing. *Genome Biology and Evolution*, **7**, 993–1001.

Labonté, J.M. & Suttle, C. A. (2013) Previously unknown and highly divergent ssDNA viruses populate the oceans. *The ISME journal*, **7**, 2169–2177.

Lachish, S., Lawson, B., Cunningham, A. a. & Sheldon, B.C. (2012) Epidemiology of the emergent disease Paridae pox in an intensively studied wild bird population. *PloS one*, **7**, e38316.

Lacorte, G.A., Félix, G.M.F., Pinheiro, R.R.B., Chaves, A. V., Almeida-Neto, G., Neves, F.S., Leite, L.O., Santos, F.R. & Braga, É.M. (2013) Exploring the diversity and distribution of Neotropical avian malaria parasites – a molecular survey from Southeast Brazil. *PLoS ONE*, **8**, e57770.

Lanfear, R., Calcott, B., Kainer, D., Mayer, C. & Stamatakis, A. (2014) Selecting optimal partitioning schemes for phylogenomic datasets. *BMC evolutionary biology*, **14**, 1–14.

Lawson, B., Lachish, S., Colvile, K.M., Durrant, C., Peck, K.M., Toms, M.P., Sheldon, B.C. & Cunningham, A.A. (2012) Emergence of a novel avian pox disease in British tit species. *PloS one*, **7**, e40176.

Lee, L.H. & Lee, K.H. (1997) Application of the polymerase chain reaction for the diagnosis of fowl poxvirus infection. *Journal of Virological Methods*, **63**, 113–119.

Lefeuvre, P., Lett, J.-M., Varsani, A. & Martin, D.P. (2009) Widely conserved recombination patterns among single-stranded DNA viruses. *Journal of virology*, **83**, 2697–2707.

Lefeuvre, P. & Moriones, E. (2015) Recombination as a motor of host switches and virus emergence: geminiviruses as case studies. *Current Opinion in Virology*, **10**, 14–19.

Levine, J.M. & HilleRisLambers, J. (2009) The importance of niches for the maintenance of species diversity. *Nature*, **461**, 254–257.

Li, L., Kapoor, A., Slikas, B., Bamidele, O.S., Wang, C., Shaukat, S., Masroor, M.A., Wilson, M.L., Ndjango, J.-B.N., Peeters, M., Gross-Camp, N.D., Muller, M.N., Hahn, B.H., Wolfe, N.D., Triki, H., Bartkus, J., Zaidi, S.Z. & Delwart, E. (2010) Multiple diverse circoviruses infect farm animals and are commonly found in human and chimpanzee feces. *Journal of Virology*, **84**, 1674–1682.

Linlin, L., Victoria, J.G., Wang, C., Jones, M., Fellers, G.M., Kunz, T.H. & Delwart, E. (2010) Bat guano virome: predominance of dietary viruses from insects and plants plus novel mammalian viruses. *Journal of Virology*, **84**, 6955–6965.

Le Loc'h, G., Bertagnoli, S. & Ducatez, M.F. (2015) Time scale evolution of avipoxviruses. *Infection, genetics and evolution*, **35**, 75–81.

Longdon, B., Brockhurst, M.A., Russell, C.A., Welch, J.J. & Jiggins, F.M. (2014) The evolution and genetics of virus host shifts. *PLoS Pathogens*, **10**, e1004395.

Maddison, W.P. & Maddison, D. (2011) Mesquite: a modular system for evolutionary analysis. Version 2.75.

Manarolla, G., Pisoni, G., Sironi, G. & Rampin, T. (2010) Molecular biological characterization of avian poxvirus strains isolated from different avian species. *Veterinary microbiology*, **140**, 1–8.

Mantilla, J.S., González, A.D., Lotta, I.A., Moens, M.A.J., Pacheco, M.A., Escalante, A.A., Valkiūnas, G., Moncada, L.I., Pérez-Tris, J. & Matta, N.E. (2016) *Haemoproteus erythrogravidus* n. sp. (Haemosporida, Haemoproteidae): Description and molecular

characterization of a widespread blood parasite of birds in South America. *Acta Tropica*, **159**, 83–94.

Martin, D.P., Biagini, P., Lefeuvre, P., Golden, M., Roumagnac, P. & Varsani, A. (2011) Recombination in eukaryotic single stranded DNA viruses. *Viruses*, **3**, 1699–1738.

Martínez-de la Puente, J., Figuerola, J. & Soriguer, R. (2015) Fur or feather? Feeding preferences of species of *Culicoides* biting midges in Europe. *Trends in parasitology*, **31**, 16–22.

Mata, V.A., da Silva, L.P., Lopes, R.J. & Drovetski, S. V. (2015) The Strait of Gibraltar poses an effective barrier to host-specialised but not to host-generalised lineages of avian Haemosporidia. *International Journal for Parasitology*, **45**, 1–10.

McCallum, H.I. (2015) Lose biodiversity, gain disease. *Proceedings of the National Academy of Sciences*, **112**, 8523–8524.

Mcclure, H.E. (1989) Epizootic lesions of house finches in ventura county, California. *Journal of Field Ornithology*, **60**, 421–430.

McGuire, J.A.A., Witt, C.C., Remsen, J.V. V, Corl, A., Rabosky, D.L.L., Altshuler, D.L. & Dudley, R. (2014) Molecular phylogenetics and the diversification of hummingbirds. *Current biology*, **24**, 910–916.

McPeek, M.A. (1996) Trade-offs, food web structure, and the coexistence of habitat specialists and generalists. *The American Naturalist*, **148**, 124–138.

Medeiros, M.C.I., Ellis, V.A. & Ricklefs, R.E. (2014) Specialized avian Haemosporida trade reduced host breadth for increased prevalence. *Journal of evolutionary biology*, **27**, 2520–2528.

Medeiros, M.C.I., Hamer, G.L. & Ricklefs, R.E. (2013) Host compatibility rather than vector-host-encounter rate determines the host range of avian *Plasmodium* parasites. *Proceedings of the Royal Society B Biological Sciences*, **280**, 20122947.

Medina, F.., Ramírez, A.G. & Hernández, A. (2004) Avian pox in white-tailed laurel-pigeons from the Canary Islands. *Journal of wildlife diseases*, **40**, 351–355.

Merino, S., Moreno, J., Sanz, J.J. & Arriero, E. (2000) Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proceedings of the Royal Society B Biological Sciences*, **267**, 2507–2510.

Merino, S., Moreno, J., Vásquez, R.A., Martínez, J., Sánchez-monsálvez, I., Estades, C.F., Ippi, S., Sabat, P., Rozzi, R. & Mcgehee, S. (2008) Haematozoa in forest birds from southern Chile: latitudinal gradients in prevalence and parasite lineage richness. *Australian Ecology*, **33**, 329–340.

Mészáros, B., István, S. & Dosztányi, Z. (2009) Prediction of protein binding regions in disordered proteins. *PLoS Computational Biology*, **5**, e1000376.

Moens, M.A.J. & Pérez-Tris, J. (2016) Discovering potential sources of emerging pathogens: South America is a reservoir of generalist avian blood parasites. *International Journal for Parasitology*, **46**, 41–49.

Mogollón, H. & Guevara, J. (2004) *Caracterización Vegetal de La Bioreserva Del Cóndor*. Fundación Numashir.

Møller, A.P., Christe, P. & Garamszegi, L.Z. (2005) Coevolutionary arms races: increased host immune defense promotes specialization by avian fleas. *Journal of evolutionary biology*, **18**, 46–59.

Montoya, J.M., Pimm, S.L. & Solé, R. V. (2006) Ecological networks and their fragility. *Nature*, **442**, 259–264.

Morand, S., Krasnov, B.R. & Littlewood, T.D.J. (2015) *Parasite Diversity and Diversification: Evolutionary Ecology Meets Phylogenetics*. Cambridge University Press, Cambridge, United Kingdom.

Muhire, B.M., Varsani, A. & Martin, D.P. (2014) SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. *PLoS ONE*, **9**, e108277.

Myers, N., Mittermeier, R.A., Mittermeier, C.G., Fonseca, G.A.B. & Kent, J. (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853–858.

Njabo, K.Y., Cornel, A.J., Bonneaud, C., Toffelmier, E., Sehgal, R.N.M., Valkiūnas, G., Russell, A.F. & Smith, T.B. (2011) Nonspecific patterns of vector, host and avian malaria parasite associations in a central African rainforest. *Molecular Ecology*, **20**, 1049–1061.

Novotny, V., Basset, Y., Miller, S.E., Weiblen, G.D., Bremer, B., Cizek, L. & Drozd, P. (2002) Low host specificity of herbivorous insects in a tropical forest. *Nature*, **416**, 841–844.

Novotny, V., Drozd, P., Miller, S.E., Kulfan, M., Janda, M., Basset, Y. & Weiblen, G.D. (2006) Why are there so many species of herbivorous insects in the tropics? *Science*, **313**, 1115–1118.

Obradovic, Z., Peng, K., Vucetic, S., Radivojac, P., Brown, C.J. & Dunker, A.K. (2003) Predicting intrinsic disorder from amino acid sequence. *Proteins: Structure, Function, and Bioinformatics*, **53**, 566–572.

Offerman, K., Carulei, O., Gous, T.A., Douglass, N. & Williamson, A.-L. (2013) Phylogenetic and histological variation in avipoxviruses isolated in South Africa. *The Journal of general virology*, **94**, 2338–2351.

Olias, P., Wegelin, M., Zenker, W., Freter, S., Gruber, A.D. & Klopfleish, R. (2011) Avian malaria deaths in parrots, Europe. *Emerging infectious diseases*, **17**, 950–952.

Opazo, J.C., Soto-Gamboa, M. & Fernandez, M.J. (2005) Cell size and basal metabolic rate in hummingbirds. *Revista Chilena de Historia Natural*, **78**, 261–265.

Ostfeld, R.S. & Keesing, F. (2012) Effects of host diversity on infectious disease. *Annual Review of Ecology, Evolution, and Systematics*, **43**, 157–182.

Parker, P.G., Buckles, E.L., Farrington, H., Petren, K., Whiteman, N.K., Ricklefs, R.E., Bollmer, J.L. & Jiménez-Uzcátegui, G. (2011) 110 years of *Avipoxvirus* in the Galapagos Islands. *PloS one*, **6**, e15989.

Pérez-Rodríguez, A., Fernández-González, S., De la Hera, I. & Pérez-Tris, J. (2013a) Finding the appropriate variables to model the distribution of vector-borne parasites with different environmental preferences: climate is not enough. *Global Change Biology*, **19**, 3245–3253.

Pérez-Rodríguez, A., Ramírez, Á., Richardson, D.S. & Pérez-Tris, J. (2013b) Evolution of parasite island syndromes without long-term host population isolation: parasite dynamics in Macaronesian blackcaps Sylvia atricapilla. *Global Ecology and Biogeography*, **22**, 1272–1281.

Pérez-Tris, J. & Bensch, S. (2005) Diagnosing genetically diverse avian malarial infections using mixed-sequence analysis and TA-cloning. *Parasitology*, **131**, 15–23.

Peréz-Tris, J., Hasselquist, D., Hellgren, O., Križanauskienė, A., Waldenström, J. & Bensch, S. (2005) What are malaria parasites? *Trends in parasitology*, **21**, 207–209.

Pérez-Tris, J., Hellgren, O., Križanauskienė, A., Waldenström, J., Secondi, J., Bonneaud, C., Fjeldså, J., Hasselquist, D. & Bensch, S. (2007) Within-host speciation of malaria parasites. *PLoS ONE*, **2**, e235.

Pérez-Tris, J., Williams, R.A.J., Abel-Fernández, E., Barreiro, J., Conesa, J.J., Figuerola, J., Martinez-Martínez, M., Ramírez, Á. & Benítez, L. (2011) A multiplex PCR for detection of poxvirus and *Papillomavirus* in cutaneous warts from live birds and museum skins. *Avian diseases*, **55**, 545–553.

Phenix, K. V, Weston, J.H., Ypelaar, I., Lavazza, A., Smyth, J.A., Todd, D., Wilcox, G.E. & Raidal, S.R. (2001) Nucleotide sequence analysis of a novel circovirus of canaries and its relationship to other members of the genus *Circovirus* of the family *Circoviridae*. *The Journal of general virology*, **82**, 2805–2809.

Pigeault, R., Ve, J., Nicot, A., Perret, P., Gandon, S. & Rivero, A. (2015) Avian malaria: a new lease of life for an old experimental model to study the evolutionary ecology of Plasmodium. *Philosophical Transactions B*, **370**, 20140300.

Pohle, P. (2008) Indigenous land use practices and biodiversity conservation in southern Ecuador. *Biodiversity and Ecological Series*, **2**, 163–176.

Poulin, R. (1999) The intra- and interspecific relationships between abundance and distribution in helminth parasites of birds. *Journal of Animal Ecology*, **68**, 719–725.

Poulin, R. (2006) *Evolutionary Ecology of Parasites (Second Edition)*. Princeton University Press.

Poulin, R. & Keeney, D.B. (2008) Host specificity under molecular and experimental scrutiny. *Trends in parasitology*, **24**, 24–28.

Poulin, R., Krasnov, B.R. & Mouillot, D. (2011) Host specificity in phylogenetic and geographic space. *Trends in parasitology*, **27**, 355–361.

Poulin, R. & Mouillot, D. (2003) Parasite specialization from a phylogenetic perspective: a new index of host specificity. *Parasitology*, **126**, 473–480.

Pushker, R., Mooney, C., Davey, N.E., Jacqué, J.-M. & Shields, D.C. (2013) Marked variability in the extent of protein disorder within and between viral families. *PloS one*, **8**, e60724.

Råberg, L., Graham, A.L. & Read, A.F. (2009) Decomposing health: tolerance and resistance to parasites in animals. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **364**, 37–49.

Råberg, L., Sim, D. & Read, A.F. (2007) Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science*, **318**, 812–814.

Rahbek, C., Bloch, H., Poulsen, M. & Rasmussen, J.F. (1995) The avifauna of the Podocarpus National Park- The "Andean jewel in the crown" of Ecuador's protected areas. *Ornitologia Neotropical*, **6**, 113–120.

Rambaut, A. & Drummond, A.J. (2007) Tracer: MCMC Trace Analysis Tool.

Rector, A., Bossart, G.D., Ghim, S.-J., Sundberg, J.P., Jenson, A.B. & Van Ranst, M. (2004) Characterization of a novel close-to-root papillomavirus from a Florida manatee by using multiply primed rolling-circle amplification: Trichechus manatus latirostris papillomavirus type 1. *Journal of virology*, **78**, 12698–12702.

Remsen, J.V., Remsen, J.R., Cadena, C.D., Jaramillo, A., Nores, M., Pacheco, J.F., Pérez-Eman, J., Robbins, M.B., Stiles, F.G., Stotz, D.F. & Zimmer, K.J. (2016) A classification of the bird

species of South America.American Ornithologists' Union

Restall, R., Rodner, C. & Lentino, M. (2007) *Birds of Northern South America: An Identification Guide, Volume 2: Field Guide*. Yale University Press.

Ricklefs, R.E. (2010) Evolutionary diversification, coevolution between populations and their antagonists, and the filling of niche space. *Proceedings of the National Academy of Sciences*, **107**, 1265–1272.

Ricklefs, R.E. & Fallon, S.M. (2002) Diversification and host switching in avian malaria parasites. *Proceedings of the Royal Society of London. Series B.*, **269**, 885–892.

Ricklefs, R.E., Fallon, S.M. & Bermingham, E. (2004) Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. *Systematic Biology*, **53**, 111–119.

Ricklefs, R.E. & Outlaw, D.C. (2010) A molecular clock for malaria parasites. *Science*, **329**, 226–229.

Ricklefs, R.E., Outlaw, D.C., Svensson-Coelho, M., Medeiros, M.C.I., Ellis, V. a. & Latta, S. (2014a) Species formation by host shifting in avian malaria parasites. *Proceedings of the National Academy of Sciences*, **111**, 14816–14821.

Ricklefs, R.E., Outlaw, D.C., Svensson-Coelho, M., Medeiros, M.C.I., Ellis, V. a. & Latta, S.C. (2014b) Diversification and host switching in avian malaria parasites. *Proceedings of the National Academy of Sciences of the United States of America*, 1–6.

Ricklefs, R.E., Swanson, B.L., Fallon, S.M., Martínez-Abraín, A., Scheuerlein, A., Gray, J., Latta, S.C., Martínez-Abraín, M., Scheuerlein, A., Gray, J. & Latta, S.C. (2005) Community relationships of avian malaria parasites in Southern Missouri. *Ecological Monographs*, **75**, 543–559.

Ridgely, R.S. & Greenfield, J.P. (2006) *Aves Del Ecuador: Guia de Campo- Volumen I & II*. Fundación Jocotoco. Colibri Digital.

Ringler, M., Mangione, R., Pašukonis, A., Rainer, G., Felling, J., Kronaus, H., Réjou-méchain, M., Reiter, K. & Ringler, E. (2014) High-resolution forest mapping for behavioural studies in the Nature Reserve " Les Nouragues ", French Guiana. *Journal of Maps*, **12**, 1–8.

van Riper III, C. & Forrester, D. (2007) Avian pox. *Infectious diseases of wild birds.*, pp. 136–176. Oxford, UK: Wiley Blackwell.

van Riper III, C., van Riper, S., Goff, L. & Laird, M. (1986) The epizootiology and ecological significance of malaria in hawaiian land birds. *Ecological Monographs*, **56**, 327–344.

van Riper III, C., van Riper, S. & Hansen, W.R. (2002) Epizootiology and effect of avian pox on Hawaiian forest birds. *The Auk*, **119**, 929–942.

Rohde, K. (2005) *Nonequilibrium Ecology*. Cambridge University Press, Cambridge, United Kingdom.

de Roode, J.C., Helinski, M.E.H., Anwar, M.A. & Read, A.F. (2005) Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. *The American Naturalist*, **166**, 531–542.

Rosario, K., Duffy, S. & Breitbart, M. (2012) A field guide to eukaryotic circular single-stranded DNA viruses: insights gained from metagenomics. *Archives of virology*, **157**, 1851–1871.

Rosario, K., Schenck, R.O., Harbeitner, R.C., Lawler, S.N. & Breitbart, M. (2015) Novel circular single-stranded DNA viruses identified in marine invertebrates reveal high sequence diversity

and consistent predicted intrinsic disorder patterns within putative structural proteins. *Frontiers in Microbiology*, **6**, 1–13.

Rudolf, V.H.W. & Antonovics, J. (2005) Species coexistence and pathogens with frequency-dependent transmission. *The American naturalist*, **166**, 112–118.

Salkeld, D.J., Padgett, K.A. & Jones, J.H. (2013) A meta-analysis suggesting that the relationship between biodiversity and risk of zoonotic pathogen transmission is idiosyncratic. *Ecology letters*, **16**, 679–686.

Santander, T., Freile, J.F. & Loor-Vela, S. (2009) Priority sites for biodiversity conservation. Ecuador. *Important Bird Areas Americas - Priority sites for biodiversity conservation.* (eds C. Devenish, D.C. Díaz Fernández, R.P. Clay, L. Davidson & I. Yépez Zabala), pp. 187–196. Birdlife International.

Schluter, D. (2000) *The Ecology of Adaptive Radiation*. Oxford University Press.

Schmid-Hempel, P. (2011) *Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology, and Genetics*. Oxford University Press.

Schoener, E.R., Banda, M., Howe, L., Castro, I.C. & Alley, M.R. (2014) Avian malaria in New Zealand. *New Zealand veterinary journal*, **62**, 189–198.

Schrenzel, M.D., Maalouf, G.A., Keener, L.L. & Gaffney, P.M. (2003) Molecular characterization of malarial parasites in captive passerine birds. *Journal of parasitology*, **89**, 1025–1033.

Schulenberg, T.., Stotz, D.F., Lane, D.F., O'Neill, J.P. & Parker, A.T.I. (2007) *Birds of Peru*. Princeton Field Guides.

Sehgal, R.N.M. (2015) Manifold habitat effects on the prevalence and diversity of avian blood parasites. *International Journal for Parasitology: Parasites and Wildlife*, **4**, 421–430.

Sheldon, B.C. & Verhulst, S. (1996) Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in ecology & evolution*, **11**, 317–321.

Sickmeier, M., Hamilton, J.A., LeGall, T., Vacic, V., Cortese, M.S., Tantos, A., Szabo, B., Tompa, P., Chen, J., Uversky, V.N., Obradovic, Z. & Dunker, A.K. (2007) DisProt: the database of disordered proteins. *Nucleic Acids Research*, **35**, 786–793.

Smits, J.E., Tella, J.L., Carrete, M., Serrano, D. & López, G. (2005) An epizootic of avian pox in endemic short-toed larks (*Calandrella rufescens*) and Berthelot's pipits (*Anthus berthelotti*) in the Canary Islands, Spain. *Veterinary pathology*, **42**, 59–65.

Stewart, M.E., Perry, R. & Raidal, S.R. (2006) Identification of a novel circovirus in Australian ravens (*Corvus coronoides*) with feather disease. *Avian Pathology*, **35**, 86–92.

Suarez, R.K., Lighton, J.R.B., Brown, G.S. & Mathieu-Costello, O. (1991) Mitochondrial respiration in hummingbird flight muscles. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 4870–4873.

Svensson-Coelho, M., Blake, J.G., Loiselle, B.A., Penrose, A.S., Parker, P.G. & Ricklefs, R.E. (2013) Diversity, prevalence, and host specificity of avian *Plasmodium* and *Haemoproteus* in a Western Amazon assemblage. *Ornithological Monographs*, **76**, 1–47.

Svensson-Coelho, M., Ellis, V. a., Loiselle, B. a, Blake, J.G., Ricklefs, R.E. & Svensson-coelho, M. (2014) Reciprocal specialization in multihost malaria parasite communities of birds: a temperate-tropical comparison. *The American naturalist*, **184**, 624–635.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology*, **28**, 2731–2739.

Thompson, J.N. (2005) *The Geographic Mosaic of Coevolution.* The University of Chicago Press.

Todd, D. (2000) Circoviruses: immunosuppressive threats to avian species: a review. *Avian pathology*, **29**, 373–94.

Todd, D. (2004) Avian circovirus diseases: lessons for the study of PMWS. *Veterinary Microbiology*, **98**, 169–174.

Todd, D., Weston, J., Ball, N.W., Borghmans, B.J., Smyth, J.A., Gelmini, L. & Lavazza, A. (2010) Nucleotide sequence-based identification of a novel circovirus of canaries. *Avian Pathology*, **30**, 321–325.

Trible, B.R. & Rowland, R.R.R. (2012) Genetic variation of porcine circovirus type 2 (PCV2) and its relevance to vaccination, pathogenesis and diagnosis. *Virus Research*, **164**, 68–77.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Kutish, G.F. & Rock, D.L. (2004) The genome of canarypox virus. *Journal of virology*, **78**, 353–366.

Valkiūnas, G. (2005) *Avian Malaria Parasites and Other Haemosporidia*. CRC Press.

Valkiūnas, G., Iezhova, T.A., Loiseau, C. & Sehgal, R.N.M. (2009) Nested cytochrome b polymerase chain reaction diagnostics detect sporozoites of hemosporidian parasites in peripheral blood of naturally infected birds. *Journal of Parasitology*, **95**, 1512–1515.

Valkiūnas, G., Palinauskas, V., Ilgūnas, M., Bukauskaitė, D., Dimitrov, D., Bernotienė, R., Zehtindjiev, P., Ilieva, M. & Iezhova, T.A. (2014) Molecular characterization of five widespread avian haemosporidian parasites (Haemosporida), with perspectives on the PCR-based detection of haemosporidians in wildlife. *Parasitology Research*, **113**, 2251–2263.

Vargas, F.H. (1987) Frequency and effect of pox-like lesions in Galapagos mockingbirds. *Journal of Field Ornithology*, **58**, 101–102.

Vazquez, D.P., Poulin, R., Krasnov, B.R. & Shenbrot, G.I. (2005) Species abundance and the distribution of specialization in host-parasite interaction networks. *Journal of Animal Ecology*, **74**, 946–955.

Waldenström, J., Bensch, S., Hasselquist, D. & Ostman, O. (2004) A new nested polymerase chain reaction method very efficient in detecting Plasmodium and Haemoproteus infections from avian blood. *The Journal of parasitology*, **90**, 189–191.

Wang, L. & Brown, S.J. (2006) BindN: a web-based tool for efficient prediction of DNA and RNA binding sites in amino acid sequences. *Nucleic Acids Research*, **34**, 243–248.

Webby, R.J. & Webster, R.G. (2001) Emergence of influenza A viruses. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **356**, 1817–1828.

Weinstein, B.G., Tinoco, B., Parra, J.L., Brown, L.M., McGuire, J.A.A., Stiles, F.G. & Graham, C.H. (2014) Taxonomic, phylogenetic, and trait beta diversity in South American hummingbirds. *The American Naturalist*, **184**, 221–224.

White, E.M., Bennett, G.F. & Williams, N.A. (1979) Avian Haemoproteidae. 11. The haemoproteids of the hummingbird family Trochilidae. *Canadian journal of Zoology*, **57**, 908–913.

Wikelski, M., Foufopoulos, J., Vargas, F.H. & Snell, H. (2004) Galápagos birds and diseases:

invasive pathogens as threats for island species. *Ecology and Society*, **9**, 1–10.

Williams, R.A.J., Escudero Duch, C., Pérez-Tris, J. & Benítez, L. (2014) Polymerase chain reaction detection of avipox and avian papillomavirus in naturally infected wild birds: comparisons of blood, swab and tissue samples. *Avian pathology*, **43**, 130–134.

Woolhouse, M.E.J. & Gowtage-Sequeria, S. (2005) Host range and emerging and reemerging infectious diseases. *Emerging Infectious Diseases*, **11**, 1842–1847.

Woolhouse, M.E.J., Haydon, D.T. & Antia, R. (2005) Emerging pathogens: the epidemiology and evolution of species jumps. *Trends in ecology & evolution*, **20**, 238–244.

Xue, B., Dunbrack, R.L., Williams, R.W., Dunker, K.A. & Uversky, V.N. (2010) PONDR-FIT: a meta-predictor of intrinsically disordered amino acids. *Biochimica et Biophysica Acta*, **1804**, 996–1010.

Xue, B., Dunker, A.K. & Uversky, V.N. (2012) Orderly order in protein intrinsic disorder distribution: disorder in 3500 proteomes from viruses and the three domains of life. *Journal of Biomolecular Structure and Dynamics*, **30**, 137–149.

Young, L.C. & Vanderwerf, E.A. (2008) Prevalence of avian pox virus and effect on the fledging success of Laysan Albatross. *Journal of Field Ornithology*, **79**, 93–98.

Zylberberg, M., Lee, K.A., Klasing, K.C. & Wikelski, M. (2012) Increasing avian pox prevalence varies by species, and with immune function, in Galápagos finches. *Biological Conservation*, **153**, 72–79.

Diversity and Host Specificity of Symbionts in Neotropical birds.